THE ACUTE INFLAMMATORY RESPONSE TO MYOCARDIAL INFARCTION

D BELL

BSc, MB, ChB, MRCP (UK)

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Department of Medicine
The Royal Infirmary
EDINBURGH

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FORMAL DECLARATION

I declare that I have written the dissertation presented to the University of Edinburgh for the degree of Doctor of Medicine; that it is based on my own observation and that, except as indicated in the thesis, the data were collected, analysed and interpreted by me.

D BELL

UNIVERSITY OF EDINBURGH

ABSTRACT OF THESIS (Regulation 7.9)

Name of Candidate DR DEREK BELL

Address Department of Thoracic Medicine, Central Middlesex Hospital, Acton Lane, London

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Experimental observations implicate the neutrophil in secondary myocardial injury following myocardial infarction and in reperfusion injury after successful thrombolysis. Activated neutrophils can induce local tissue injury through the release of proteolytic enzymes and via the generation of oxygen derived free radicals, but are also involved in the no-reflow phenomena seen after reperfusion. The object of this thesis was therefore to investigate the role of the neutrophil in myocardial infarction in man.

A method for studying the neutrophil uptake in damaged myocardium was developed which allowed rapid separation and subsequent radiolabelling of a "pure" population of autologous neutrophils using Indium-111 oxine (^{111}In). ^{111}In-labelled neutrophils exhibit normal in-vivo kinetics, with rapid lung clearance and migrate to sites of infection and inflammation and were used to image the acute inflammatory infiltrate in 30 patients with acute infarction. Imaging success was dependent on time of injection of the cells in relation to onset of symptoms. All patients injected within 18 hours had positive images whereas less than 50% of patients injected after 24 hours had positive images. The use of gamma camera imaging with single photon emission computed tomography improved imaging and allowed quantification of myocardial uptake of radioisotope. A subsequent study showed that neutrophil uptake was less extensive, relative to infarct size (assessed by Technetium-99m pyrophosphate) in patients treated with thrombolytic therapy compared to patients not given thrombolysis.

A further study investigated neutrophil activation, by measuring plasma neutrophil elastase, and free radical production, by measuring the non-peroxide diene conjugated isomer of linoleic acid (PL-9, 11-LA') in 32 patients with acute myocardial infarction, 30 normal subjects and 35 patients with stable ischaemic heart disease. Neutrophil elastase was measured using a standard radioimmunoassay and a method established for measuring PL-9, 11-LA' by high performance liquid chromotography. Samples taken over the 48 hours following myocardial infarction showed neutrophil elastase was significantly increased compared to normals or patients with ischaemic heart disease. Patients not treated with thrombolytic therapy had a late increase in neutrophil elastase at 40 hours, consistent with release from activated neutrophils present within injured myocardium. In contrast, patients treated with thrombolytic therapy had increased levels of neutrophil elastase at 8 hours consistent with intravascular neutrophil activation. Plasma PL-9, 11-LA'was significantly increased in patients with acute myocardial infarction compared to normal subjects or patients with ischaemic heart disease. The levels were similar in patients treated with and without thrombolytic therapy.

The results confirm neutrophil migration to the site of myocardial infarction with neutrophil activation and free radical production. However, they do not suggest that reperfusion is a significant clinical problem, as thrombolytic therapy appears to reduce rather than enhance the acute inflammatory infiltrate and is not associated with a greater increase in neutrophil activation or free radical production. Further modification of the inflammatory response is unlikely to be of benefit following thrombolytic therapy, but may be a method to improve myocardial salvage in patients not eligible for thrombolysis.

INDEX OF CONTENTS

THE PURPOSE OF THIS THESIS

In most industrialised countries, ischaemic heart disease is the commonest cause of death, and accounts for approximately 30% of all deaths in the United Kingdom. The syndrome of myocardial infarction, though only recognised as recently as 1912, now represents 15-20 percent of all medical admissions to Edinburgh Royal Infirmary.

The introduction of coronary care units in the 1960's led to an improved understanding of the evolution and complications of acute myocardial infarction, followed by subsequent improvement of in-hospital mortality by early, effective treatment of life threatening arrhythmias. The major problems in coronary care then became severe left ventricular failure, cardiogenic shock and extension of infarction. It has now been established that following myocardial infarction, the main determinant of morbidity and mortality both in the acute phase and for survivors is infarct size and associated residual left ventricular function. This has led to attempts to preserve myocardial function, particularly using thrombolytic drugs to recanalise and hence reperfuse the infarct related coronary artery. Recent studies have shown the clinical benefit of this therapy which reduces mortality and preserves ventricular function, particularly in patients

with anterior myocardial infarction. In general, coronary care is of most benefit to patients admitted early as most of the life threatening complications of myocardial infarction occur in the first hours following the onset of symptoms. Likewise, thrombolytic therapy appears to be of greatest benefit when administered within 4 hours and preferably within 1 hour of the onset of symptoms. In practical terms this means that functional myocardial salvage is most likely to be achieved in patients admitted early in the course of myocardial infarction. This means that for many patients only symptomatic treatment is available.

Despite the increasing clinical evidence showing the beneficial effects of thrombolysis, there is considerable experimental evidence which suggests that the restoration of a blood supply to an ischaemic area of myocardium may cause the death of irreversibly injured myocytes, so-called reperfusion injury. Reperfusion produces a complex group of phenomena some of which have been implicated in accelerating myocardial damage. These include the no-reflow phenomenon which is largely due to capillary plugging by neutrophils, calcium influx, the production of oxygen derived free radicals and the release of proteolytic enzymes from phagocytes. Therefore the neutrophil, may be an important component of reperfusion injury, as this cell is involved in the no-reflow

phenomena, is a major source of proteolytic enzymes and is capable of generating oxygen free radicals as well as generating mediators of the inflammatory response.

The neutrophil may also be important in secondary myocardial injury following acute myocardial infarction in patients who have not received thrombolytic therapy. In common with all tissue damage, myocardial infarction elicits a normal local acute inflammatory response with early neutrophil migration, at the site of tissue injury. As such, the neutrophils represent an essential component of the inflammatory response being necessary for phagocytosis and removal of necrotic tissue allowing subsequent tissue remodelling. However, experimental studies have shown that neutrophil depletion of animals prior to occlusion of a coronary artery can reduce the size of the infarct. Thus, it is possible that therapy designed to regulate the inflammatory response to myocardial infarction, may improve myocardial salvage in patients treated with or without thrombolytic agents.

The purpose of this thesis was therefore to examine the potential role of the inflammatory response and in particular the neutrophil, in myocardial infarction and also to study the effect of thrombolytic therapy on this response. Post mortem and animal studies have shown that neutrophils are present within the area of myocardial

necrosis within 24 hours of infarction and continue to migrate over the next 48-72 hours. This response can be imaged using radiolabelled autologous neutrophils, which although normally used to detect sites of occult infection, have also been used to image acute inflammation. White cell imaging has been used to detect the acute inflammatory response at the site of myocardial infarction in dogs and in one previous study in man, it was hoped to use this technique to further study the role of the neutrophil in myocardial infarction in man. First it was necessary to establish a method for reliably separating and radiolabelling a "pure neutrophil" population from blood obtained from patients with acute myocardial infarction. After re-injection, the radiolabelled autologous neutrophils were used to study the neutrophil uptake at the site of myocardial injury using conventional gamma camera imaging. Factors which could potentially influence this response, such as infarct size and time from onset of symptoms, were also studied. The extent or volume of uptake of radiolabelled neutrophils was quantified using single photon emission computed tomography and compared with infarct size, assessed by the same method, using the infarct avid imaging agent Technetium-99m pyrophosphate. The effect of thrombolytic therapy on the inflammatory infiltrate in relation to infarct size was studied to assess the effect of reperfusion.

In the second part of the study, two possible mechanisms by which neutrophils could cause secondary myocardial injury were investigated. Activated neutrophils release a number of potent proteolytic enzymes, amongst which is neutrophil elastase. This enzyme has not only been implicated in the pathogenesis of a number of diseases, but can also be measured in plasma, using a sensitive radioimmunoassay, and provides a useful marker of neutrophil activation. Plasma neutrophil elastase was therefore measured in serial blood samples taken from patients in the first 48 hours of myocardial infarction to study the extent of neutrophil activation and compared with the appearance in plasma of the cardiac associated enzyme creatine kinase.

Another mechanism by which activated neutrophils may extend myocardial injury is through the release of oxygen free radicals, though neutrophils are not the only potential source of free radical production within ischaemic myocardium. Measuring free radical activity in vivo is difficult, and the methods are usually indirect, because of the short biological half-life of microseconds. The most studied methods measure the auto-oxidation products of polyunsaturated fatty acids, a process termed lipid peroxidation or lipid oxidation. These compounds are commonly used to measure free radical activity, but

some have also been directly implicated in cellular injury by producing membrane damage. Recently the non-peroxide diene conjugated isomer of linoleic acid has been identified as the major diene conjugate in human plasma and is thought to reflect free radical production. Using high performance liquid chromatography this isomer was also measured in serial blood samples taken from patients in the first 48 hours of myocardial infarction. In addition, to assessing the pattern of appearance of neutrophil elastase and the diene conjugate of linoleic acid following myocardial infarction, the effect of thrombolytic therapy on these parameters was studied and the results also compared with normal control subjects and patients with stable ischaemic heart disease.

Using the three methods described, further information about the pattern of events following myocardial infarction was established both in patients treated with and without thrombolysis.

The aim of this study was, therefore, to further elucidate the role of the neutrophil in myocardial infarction and study potential mechanisms of secondary myocardial injury. This knowledge may be of therapeutic benefit as regulation of the inflammatory response, for example, using non-steroidal anti-inflammatory drugs or free radical scavengers may reduce secondary myocardial damage, thus

providing another method for preserving myocardial function following acute myocardial infarction in man.

CHAPTER 1

HISTORICAL BACKGROUND AND MANAGEMENT OF ACUTE MYOCARDIAL INFARCTION

Fatal coronary atherosclerosis and thrombosis is now the commonest cause of death in the Western World. Although atheroma has been described in man for many centuries, the clinical manifestations of coronary artery disease have only become evident in the last three centuries. Angina pectoris was first described by William Heberden in his "Some account of a Disorder of the Breast" 1772 in which he wrote "Those who are afflicted with it are seized while they are walking, and more particularly when they walk soon after eating, with a painful and most disagreeable sensation in the heart which seems as if it would take their life away, if it were to continue: the moment they stand still, all this uneasiness vanishes".

Despite this excellent clinical description it was not until 1799 that Parry expressed the concept of myocardial ischaemia, relating abnormal coronary blood supply to demand, and not until 1809 that Burns suggested that cardiac pain was a consequence of ischaemia. It was then a further century before Herrick clearly described the association between coronary artery occlusion and

myocardial infarction (Herrick, 1912). In a later publication, Herrick recognised four groups of patients with myocardial infarction; those in the first two groups deteriorated rapidly and died soon after the onset of symptoms. A third group in whom death was delayed for hours, days or months with the possibility of recovery and a fourth group who had mild symptoms and a better prognosis which he related to obstruction of the smallest branches of the coronary arteries (Herrick, 1918 and 1919). The electrocardiographic features of myocardial infarction were then later described (Pardee, 1925) which allowed physicians to distinguish between acute myocardial infarction and angina pectoris in life.

With the ability to diagnose acute myocardial infarction in life it became apparent that course and prognosis were so variable that each case must be considered individually. However, a number of poor prognostic features were initially identified by White in a large series from Massachusetts General Hospital (White, 1926). He concluded that the following findings made the prognosis worse: advanced age, a state of shock, an abrupt and pronounced fall in blood pressure, the prolongation of severe substernal pain for hours, the duration of fever for more than a few days, the presence of a high fever ($103^{o}F$-$104^{o}F$), a high leucocytosis, especially if maintained for a week or more, rapid and

marked cardiac dilatation, gallop rhythm, ventricular paroxysmal tachycardia, heart block, pulsus alternans, cardiac asthma, congestive cardiac failure and embolic phenomena. He also introduced the concept that prognosis depended on the speed and degree of cardiac involvement and that the duration and height of fever was related to the size of the infarct. Thereafter numerous studies were performed to establish which variables were important in determining prognosis following acute myocardial infarction, most incorporating the factors described by White which he later discussed in a larger series (White, 1932). Rosenbaum and Levine (1941) and Billings et al (1949) found that the two most important adverse prognostic features in terms of immediate outcome and long term clinical status were the presence of shock and cardiac failure, features which we now know are related to the degree of myocardial damage. However, Rosenbaum and Levine concluded that "weighing all the information available together with the general appearance of the patient enables the physician to make a fair estimate as to the immediate prognosis". Thereafter, the trend shifted from looking for a single isolated variable to the development of a weighted score, based on the severity of a number of adverse features. This led to the development of the Coronary Prognostic Index (Peel, 1962; Norris, 1969) in an attempt to predict early mortality. Though, numerous other studies have attempted

to devise prognostic indices following myocardial infarction the common factors in all studies appear directly or indirectly related to the degree of myocardial damage sustained. This concept was present in White's original observations in 1926 when he suggested that the prognosis depended on the speed and degree of myocardial involvement following coronary thrombosis. It follows that the greater the extent of myocardial infarction, the poorer the residual cardiac function with the greater likelihood of clinical evidence of cardiac failure or shock, radiological changes of pulmonary oedema, abnormal blood chemistry or leucocytosis. The concept that residual myocardial function was the most important prognostic feature of survival was confirmed by Hammermeister in a study of 733 patients with documented coronary artery disease (Hammermeister, 1979). Using univariate and multivariate analysis of a number of variables including clinical examination, resting electrocardiogram, exercise testing, coronary arteriography and quantitative left ventricular contrast angiography he found that the variable most predictive of survival was left ventricular ejection fraction. Subsequent studies have shown that invasive (Verdouw, 1975) and non-invasive (Dewhurst, 1981) assessment of left ventricular function is also of prognostic value in predicting heart failure and sudden death following acute myocardial infarction.

While the pathological basis, clinical signs, diagnostic investigations and prognostic indices of myocardial infarction were established between the 1920's and 1950's, little advance was made in therapy. Indeed the advice given in the 1920's and 1950's varied little and was all based on the relief of symptoms with particular emphasis on diet, analgesia and strict bed rest. The lack of emphasis on treatment in the intervening years was emphasised by Biorck (1960) who wrote "There are few diseases in the sphere of internal medicine where the average mortality during four to six weeks hospitalisation is over 30%, and if the patients with shock are particularly considered, the figure is more than twice as large. It is obvious that the task of treatment and prevention is tremendous and it appears necessary that more energy be directed to a considerable reduction in these figures. The mere quantity of the problem may have prevented us from calling all forces to arms in the "infarct battle". However, our surgical colleagues would never accept a mortality of this magnitude and would certainly mobilise personnel and technique to bring such figures down".

Shortly afterwards, following successful reports of cardiopulmonary resuscitation techniques in acute myocardial infarction (Julian, 1961) the first coronary

care units were established in Australia and North America. Early reports from such units were published in 1963 (Day, 1963; Brown, 1963) and emphasised the importance of arrhythmias as the cause of death. This knowledge led to a greater understanding of arrhythmias, particularly those which are life threatening, with subsequent improvement in the methods of treatment. An Edinburgh study demonstrated that the coronary care units reduced mortality to 16.4% in the first year of operation compared to 23.4% in the preceding year and that a significant proportion of patients survived treated ventricular fibrillation (Julian, 1968). Despite these benefits certain trials showed no difference in mortality between home-management and hospital management groups (Mather, 1976; Hill, 1978). These trials have been subsequently criticised because they were of insufficient power to assess effectiveness and examined low risk groups, but they did serve to demonstrate that coronary care units were of greatest benefit to high risk groups. With the early successes in treatment of primary tachyarrhythmias and conduction disorders, the main causes of death became advanced left ventricular failure and cardiogenic shock. It is now clear that to treat such patients effectively invasive haemodynamic monitoring to accurately monitor and adjust inotropic and vasodilator drug therapy is required. However, to date, particularly in cardiogenic shock, there is no evidence that final

outcome is affected by these treatments and they are costly in terms of time and resources.

Thus, it was apparent that the success of coronary care units was largely due to treatment of arrhythmias but patients were still dying from pump failure. As already discussed the evidence suggests that residual ventricular function is related to the extent of myocardial infarction which in turn relates to prognosis. Thus, patients who die from cardiogenic shock have massive infarcts (Page, 1971) whereas survivors of small infarcts tend not to develop cardiac decompensation (Sobel, 1972).

It is on this background that attempts at early intervention designed to limit infarct size have been designed. The concept owes much to the early work of Maroko and Braunwald (Maroko, 1971) who studied a number of factors which might influence infarct size following experimental coronary occlusion in dogs. They demonstrated a number of factors which increased infarct size, but also demonstrated a beneficial effect on infarct size with propranolol. The early use of beta-blockade following myocardial infarction in man has been shown to reduce indirect indices of infarct size (Yusuf, 1985) and long term beta-blockade reduces subsequent mortality and reinfarction (The Miami Trial Research Group, 1985 and ISIS-I, 1986). Many other forms of treatment

aimed at reducing infarct size have been tried, including nitrates, hyaluronidase, glucose-insulin-potassium, and corticosteroids but the only other group of drugs which have been shown to be of clinical benefit in man are the thrombolytic agents.

The thrombolytic agent streptokinase was first used in myocardial infarction by Fletcher in 1958, but despite initial promising results did not enter routine clinical use. The resurgence of the use of thrombolytic agents follows the demonstration that coronary artery occlusion leading to myocardial infarction is the result of thrombus formation (De Wood, 1980), usually at the site of a ruptured atheromatous plaque (Davies, 1985).

Studies have now shown that an intracoronary or intravenous infusion of thrombolytic drugs will result in lysis of the intracoronary thrombus with consequent reperfusion, without unacceptable bleeding problems (Schroder, 1983; Rentrop, 1985). All thrombolytic agents act as plasminogen activators and exert their fibrinolytic effect by converting plasminogen into its proteolytically active form, plasmin. Plasmin then digests the insoluble fibrin network into soluble fibrin degradation products (fig 1a). Streptokinase and urokinase are currently available for coronary thrombolysis, but two new agents designed to produce less systemic anticoagulation have

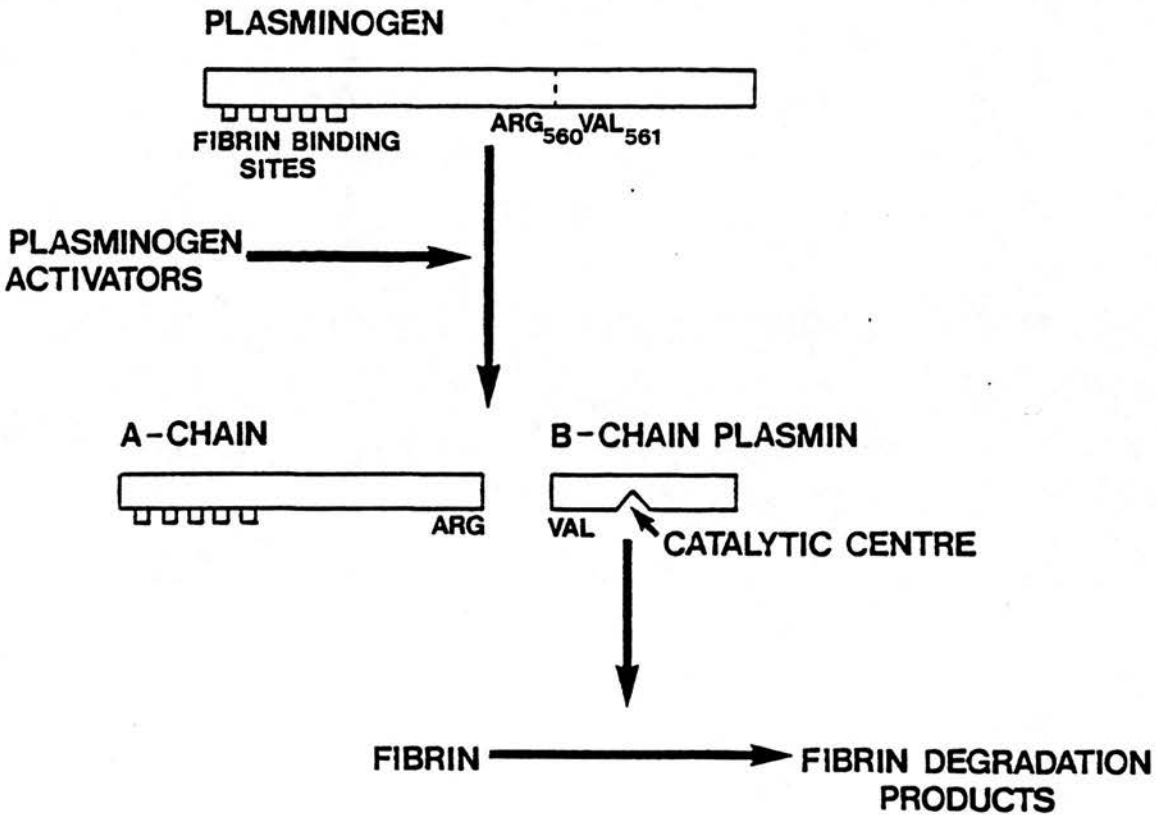


Fig 1a Diagrammatic representation of the fibrinolytic enzyme system, showing the site of action of plasminogen activators.

also been shown to be effective in clinical trials. These are tissue plasminogen activator which appears to lyse thrombus rapidly with the additional advantage of being non-antigenic (The TIMI Study Group, 1985) and anisoylated plasminogen-streptokinase activator complex (APSAC) which is effective given as a single intravenous injection (Been, 1986). Assessing the efficacy of thrombolytic therapy in terms of coronary artery patency is difficult because of spontaneous reperfusion which occurs in 20-30% of cases (De Wood, 1980). However, the patency rate with intravenous streptokinase appears to be in the range 55-75% (Neuhaus, 1981; Schroder, 1983) compared to 60-80% with intracoronary streptokinase (Khaja, 1983; Anderson, 1983). For practical purposes the intravenous route of administration is the only realistic option in view of the large numbers of patients involved, the cost and the ability to administer therapy outwith a cardiac catheterisation laboratory (Verstraete, 1985). Whilst coronary reperfusion is the immediate aim of this form of therapy the object of thrombolysis is to achieve functional myocardial salvage by reducing infarct size. The Netherlands Interuniversity Study in 533 patients demonstrated that infarct size as measured by release of cardiac associated enzyme was reduced in these patients given streptokinase, and that this effect was greater the earlier the treatment was started. Enzyme release was reduced by 51% in patients treated

within one hour, 31% in those treated between one and two hours, but only by 13% in those treated between two and four hours (Simoons, 1986). They also demonstrated improved LVEF in the treated group (Serruys, 1986). Similarly Been and co-workers demonstrated that the earlier the treatment with anisoylated plasminogen-streptokinase activator complex the more normal the LVEF, again suggesting reduction of infarct size (Been, 1986).

Two large clinical trials have now reported reduced mortality in patients treated with intravenous streptokinase as one would expect if infarct size were reduced. The Gruppo Italiano per lo Studies della Streptochinasei nell Infarts Miocardico (GISSI) reported their study of 11712 patients and have shown reduced hospital mortality for streptokinase treated patients which is maintained in the first year after infarction (Gissi, 1986; 1987). Results from the Second International Study of Infarct Survival, a trial aimed at recruiting 20,000 patients have shown reduced in hospital mortality in the streptokinase group (9%) compared to 12% in placebo allocated groups (ISIS-2, 1987; 1988). It would appear from these studies that the benefits are greatest from patients treated early, particularly within four hours and preferably within one hour.

Thus, there appears to be ever increasing evidence of the

clinical effectiveness of early thrombolysis in reducing infarct size and improving survival. Despite this clinical evidence there is still considerable debate in the literature about the possible deleterious effects of reperfusion. Early animal studies on myocardial reperfusion had predicted that potentially fatal reperfusion arrhythmias may occur which might limit the value of restoring an adequate blood supply particularly if reperfusion is delayed (Smith, 1974). Clinical experience has shown that although reperfusion arrhythmias do occur they are not a serious adverse factor limiting the use of thrombolytic drugs. Others have argued that reperfusion of ischaemic myocardium can accentuate tissue injury and have argued against widespread adoption of this therapy, particularly if given late after the onset of symptoms. Braunwald in discussing this problem spoke of myocardial reperfusion being a double edged sword with the advantages of reperfusion and reoxygenation being partly offset by the extension of cellular damage that such reperfusion may cause (Braunwald, 1985). One postulated mechanism by which reperfusion injury may result is through an exaggerated inflammatory response with release of cytotoxic products from activated neutrophils with consequent local tissue damage. Similarly, the inflammatory response has also been implicated in secondary myocardial injury following acute myocardial infarction (Romson, 1982).

The object of this thesis was to examine the role of the inflammatory response, with particular emphasis on the neutrophil, in myocardial infarction in man. Further, it was hoped to assess the effect of thrombolytic drugs on the acute inflammatory response and the potential for reperfusion injury. In order to assess this it is essential to understand the normal response to ischaemic myocardial injury as well as the potential mechanisms of reperfusion injury. These are outlined in chapter 2 along with the proposed methods for studying these phenomena in man.

CHAPTER 2

THE INFLAMMATORY RESPONSE TO MYOCARDIAL INJURY

Inflammation is best defined as the local reaction of vascular connective tissue to injury. The inflammatory response can be divided into two types, acute and chronic. In acute inflammation a succession of changes take place within a short period which may be from minutes to days: it is brought to an end either by a return of the tissue to normal function with repair by fibrosis or progression to chronic inflammation. It is important to realise that chronic inflammation is not just acute inflammation extended in time as the vascular and cellular changes are markedly different. Most of the discussion hereafter will relate to the changes seen in acute inflammation.

HISTORICAL BACKGROUND

For a long time inflammation was considered a well defined and specific disease entity characterised by the four cardinal signs, "color", "tumor", "rubor" and "dolor" (heat, swelling, redness and pain). A fifth sign "functio laesa" (loss of function) was wrongly attributed to Galen in the second century, but the real source was Virchow (Majno, 1975). It was later that John Hunter noted that inflammation was a non-specific response

(Hunter, 1794) and although this response follows a broad general pattern it can vary in detail according to the nature, site and severity of the injury. The advent of the microscope allowed Conheim to study the nature of the cellular response to injury which he described in his lectures, published in English in 1889. These gave an account of the morphological changes and demonstrated the essential part of the vascular system and leucocytes in the inflammatory response. In the same decade Metchnikoff described phagocytosis and emphasised its importance in the hosts defence to tissue injury (Metchnikoff, 1883).

The local clinical signs of acute inflammation described by Celsus and later Virchow are induced by (1) changes in the microcirculation, (2) changes in vascular permeability, and (3) the cellular exudate. These three reactions may overlap and some share common mediator mechanisms but for clarity these phases will be considered separately.

Microcirculatory changes

Immediately following injury, there is a variable but usually very short period of arteriolar vasoconstriction, which is rapidly followed by arteriolar dilation and the opening of new microvascular beds. In the immediate vicinity of the injury, blood flow then begins to slow

probably secondary to increased permeability of the microvasculature, with escape of protein rich fluids into the extravascular space. This results in increased blood viscosity and stasis of red blood cells. In addition there is greater adhesiveness and sticking of leucocytes to the endothelial cells. On the periphery of the injured area, however, blood flow remains increased.

Changes in vascular permeability

The permeability of the microvasculature is increased for most of the duration of the inflammatory response. The nature and degree of tissue injury determines the type and duration of the permeability changes. With mild injury the increase in permeability is immediate, but transient, and is due to the contraction of the endothelial cells lining venules. More severe injury, produces the same initial response which is persistent, may last 24 hours and also corresponds to contraction of venular endothelial cells. With even more severe injury, such as thermal injury, the increased permeability is result of destruction of the capillary bed. In addition to the increased permeability, the adhesiveness of the vascular aspect of endothelial cells is also increased.

Cellular exudate

At the site of injury polymorphonuclear leucocytes adhere momentarily to the endothelium and roll along the inner

surface of the vessel wall, slower than the axial stream of red blood cells, adhering briefly before they re-enter the circulation. Later greater numbers of polymorphs adhere to the endothelial surface with fewer being dislodged, a process called margination. This process is facilitated by the initial microcirculatory changes but is a more complex interaction involving changes in cell surface characteristics with mediators being derived from both leucocytes and endothelial cells, particularly products of arachidonic acid metabolism. Other mediators including components of the complement system, are also important in amplifying this response and are discussed later. The marginated polymorphs, usually neutrophils, then leave the intravascular space, initially by inserting a pseudopodium between the endothelial cells followed subsequently by the whole cell, a process called diapedesis. Ultimately they emigrate into the area of tissue damage and healing begins with phagocytosis of the necrotic tissue which is facilitated by the release of proteolytic enzymes from the activated neutrophils. Repair ultimately occurs by fibrosis.

The phases of inflammation described above are part of an elaborate homeostatic mechanism controlled by a number of chemical mediators. An inflammatory mediator being an endogenous substance whose levels increase at the site of injury in association with the appearance of at least one

tissue response or structural change. These mediators can arise from plasma, from cells or from damaged tissue and may affect more than one phase of the inflammatory response. Many chemical mediators are now thought to be important in inflammation and they have been broadly divided into the following groups:

1. Vasoactive amines - histamine, 5-hydroxytryptamine, platelet activating factor.
2. Plasma proteins:
   a) Kinins - Hageman factor, kallikrein, bradykinin
   b) Complement system (C3a, C5a)
   c) Coagulation - fibrinolytic system - fibrinogen, fibrin and fibrin degradation products.
3. Arachidonic acid metabolites:
   a) Cycloxygenase pathway (prostaglandins, thromboxane, endoperoxides)
   b) Lipoxygenase pathway (leukotriene, HETE)
   c) Chemotactic lipids
4. Lysosomal contents - cationic protein, elastase, collagenase.
5. Oxygen derived free radicals.
6. Lymphokines.
7. Monokines.
8. Others.

Although the mediators control and implement the

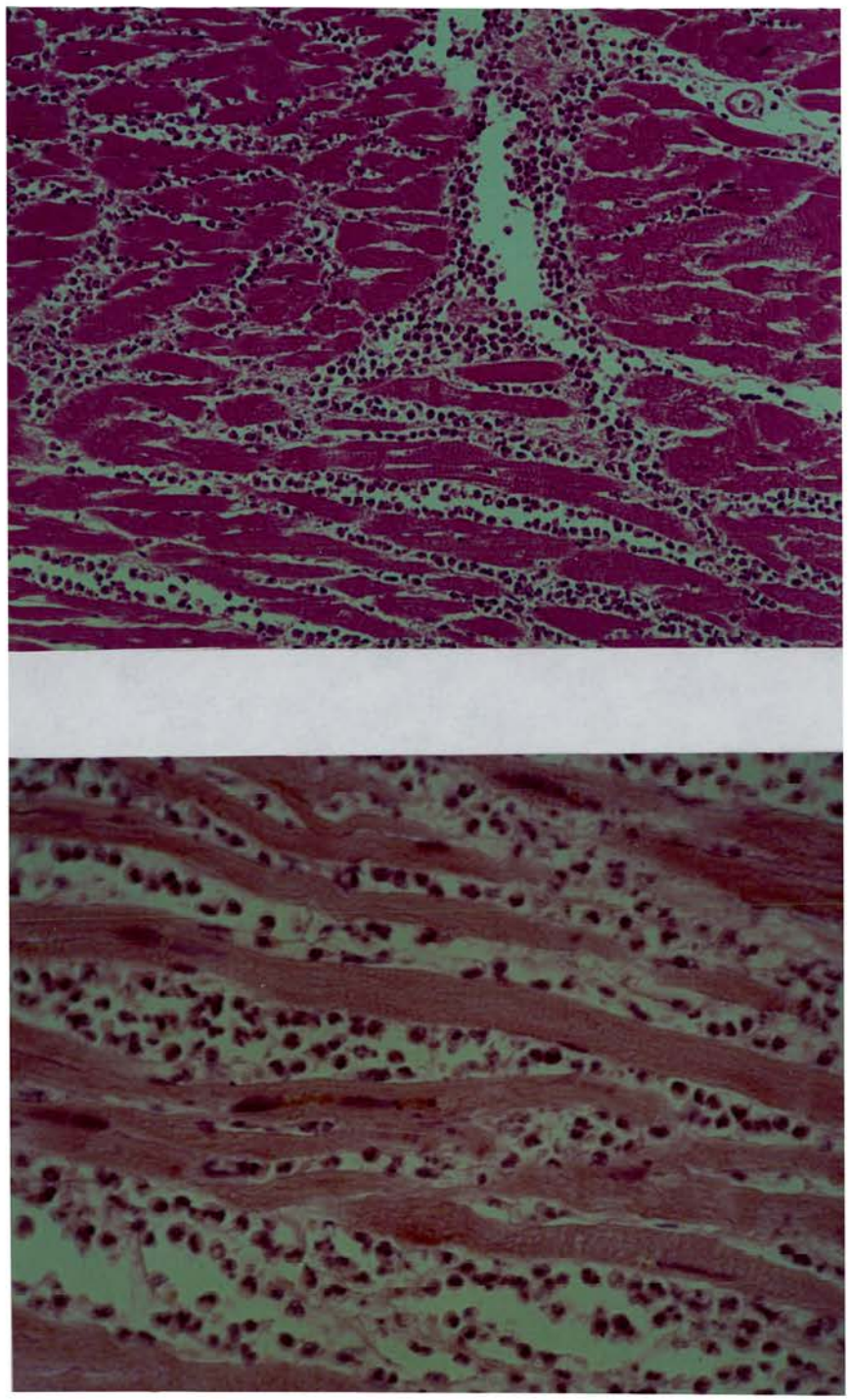
inflammatory response, it is now well established that at least some part of the tissue damage observed in acute inflammation is caused not by the inciting agent, but by the inflammatory process itself. Secondary tissue injury of this nature is probably brought about through excess or uncontrolled production of mediators, many of which may be derived from the neutrophil infiltrate (Henson, 1972; Gallin, 1984). Some of these mechanisms will be discussed more fully later in this chapter and may be important in secondary myocardial injury.

The first detailed description of the histopathological changes involved in the healing of myocardial infarction in man was published by Mallory in 1939. This was a post mortem series which predominantly examined the histological changes in the first five weeks following infarction. The description of events is very similar to those later described in experimental acute myocardial infarction in dogs (Sommers, 1964). Both studies describe the acute and chronic inflammatory response to myocardial injury with subsequent healing by fibrosis. The histological changes following infarction develop slowly with definite evidence of cell necrosis not being detectable until 4 hours. In addition to the morphological changes in the myocytes, the microcirculatory changes and the appearance of the cellular inflammatory infiltrate are also described. At

approximately 4 hours, dilatation of the blood vessels with early neutrophil margination occurs at the periphery of the infarct. By 24 hours the neutrophil infiltrate is present spreading from the periphery to the more central areas of infarction (figs 2a and 2b), is maximal by 3-4 days, and thereafter gradually diminishes before being replaced by chronic inflammatory cells. Macrophages begin to appear in the damaged areas around 4 days, at approximately the same time as granulation tissue, and begin to remove necrotic tissue. Between 6 and 10 days lymphocytes and plasma cells are present with new collagen evident at approximately 12 days.

NEUTROPHILS IN MYOCARDIAL INJURY

Though the inflammatory response is considered to be an essential part of the normal host defence and healing process following tissue injury, it is known that this response can produce tissue damage per se. Indeed the neutrophil has been implicated in the pathogenesis of a number of disease conditions (Malech, 1987) and the same oxidative and non-oxidative processes which are important in killing microorganisms have also been implicated in secondary tissue injury. The neutrophil may also have a role in secondary myocardial injury following acute myocardial infarction as well as contributing to reperfusion injury. Interest in the role of the



Figs 2a and 2b Low (fig 2a) and high power (fig 2b) photomicrographs taken from an area of myocardial infarction showing neutrophil infiltrate within the ischaemic area of myocardium.

neutrophil in myocardial injury stemmed from the initial observations which showed rapid accumulation of neutrophils in the area of myocardial infarction (Sommers, 1964), and a series of subsequent experiments have now suggested a causal association between the neutrophil and secondary myocardial injury. Romson and co-workers first demonstrated that the cardioprotective effects of the non-steroidal anti-inflammatory agent ibuprofen on infarct size were the result of reduced neutrophil infiltration in the region of the infarct (Romson, 1982). Subsequent work demonstrated that depletion of circulation neutrophils prior to experimental coronary occlusion also reduced infarct size (Romson, 1983). This work has subsequently been confirmed, but it has been suggested that the beneficial effects of neutrophil depletion are less, the greater the duration of myocardial ischaemia (Jolly, 1986). However, as the neutrophil obviously has an essential role in wound healing it would not seem desirable to totally block the local inflammatory response to myocardial ischaemia and in certain animal species such therapy may be associated with scar thinning (Jugdutt, 1985) and impaired ventricular function (Hammerman, 1983).

Activated neutrophils may produce tissue injury through the release of a number of products (Henson, 1972) and can themselves amplify the inflammatory response (Snyderman, 1981). The neutrophil can produce highly reactive and

cytotoxic oxygen species including superoxide anion, hydroxyl radical, hydrogen peroxide and singlet oxygen and such reactive species can damage extracellular macromolecules (Carrell, 1982), attack membrane phospholipids (Meerson, 1982), and disrupt normal mitochondrial function (Turrens, 1982) thus promoting cell death or injury. Another mechanism by which neutrophils may promote tissue damage is through the release of lysosomal enzymes, into the microenvironment, capable of proteolytic disruption of viable as well as irreversibly injured tissue. One of the most potent of the lysosomal enzymes is neutrophil elastase, a serine protease, whose substrates include the following important matrix molecules; elastin, collagen, proteoglycans, fibronectin and fibrinogen. This enzyme is implicated in the pathogenesis of a number of diseases, the best documented of which is probably pulmonary emphysema (Janoff, 1985).

Numerous tissue and blood derived inflammatory mediators, including complement, are involved in the initial recruitment of neutrophils to the site of myocardial injury (Maroko, 1978; Pinckard, 1975). However, in addition, stimulated neutrophils may themselves amplify this response by the release of arachidonic acid from membrane phospholipid which is converted via the lipoxygenase pathway to hydroxy-eicososatetranoic acids and leukotrienes both of which are potent chemoatractants

for neutrophils. The potential role of the neutrophil in myocardial injury is shown diagrammatically in fig 2c, and these mechanisms are similar to those postulated in the pathogenesis of a number of other diseases (Malech, 1987).

The neutrophil may also contribute to reperfusion injury following successful thrombolytic therapy. By definition reperfusion injury refers to cell death or damage resulting from restoration of the blood supply, in contrast to the cell death or damage produced by the preceding episode of ischaemia (Hearse, 1977). If reperfusion is instituted at a time when ischaemic but viable myocytes are present infarct size is limited (Reimer, 1979). Jennings first suggested that reperfusion hastened the necrosis of some irreversibly injured myocytes (Jennings, 1960) and several mechanisms have now been proposed which may contribute to reperfusion injury. These include (1) failure of the blood flow to return uniformly to the ischaemic area, termed the no-reflow phenomena which is probably due to the plugging of the microvascular bed by neutrophils (Ames, 1968; Kloner, 1974), (2) the influx of calcium following reperfusion leads to tissue disruption (Hearse, 1977), (3) oxygen derived free radicals (McCord, 1985; Werns, 1986), and (4) neutrophil release products (Braunwald, 1985). It can therefore be seen that the neutrophil may have a key role in reperfusion injury (fig 2d). The "no-reflow

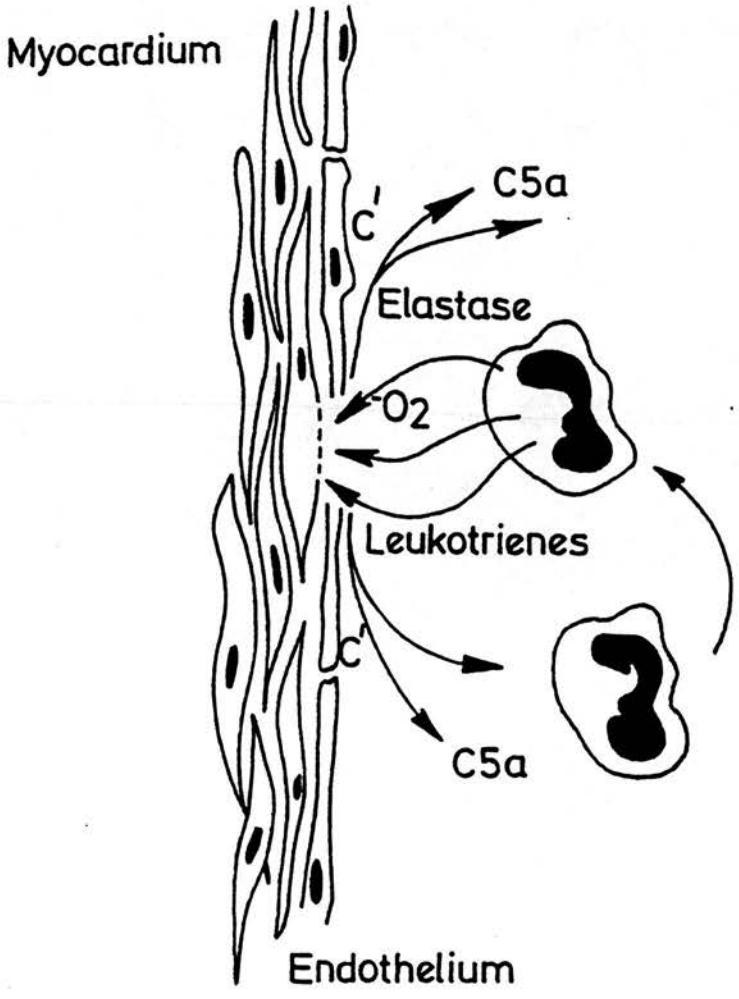


Fig 2c Diagram outlining some of the mechanisms of neutrophil mediated myocardial injury showing activation of the complement system (C^1) by damaged myocytes.

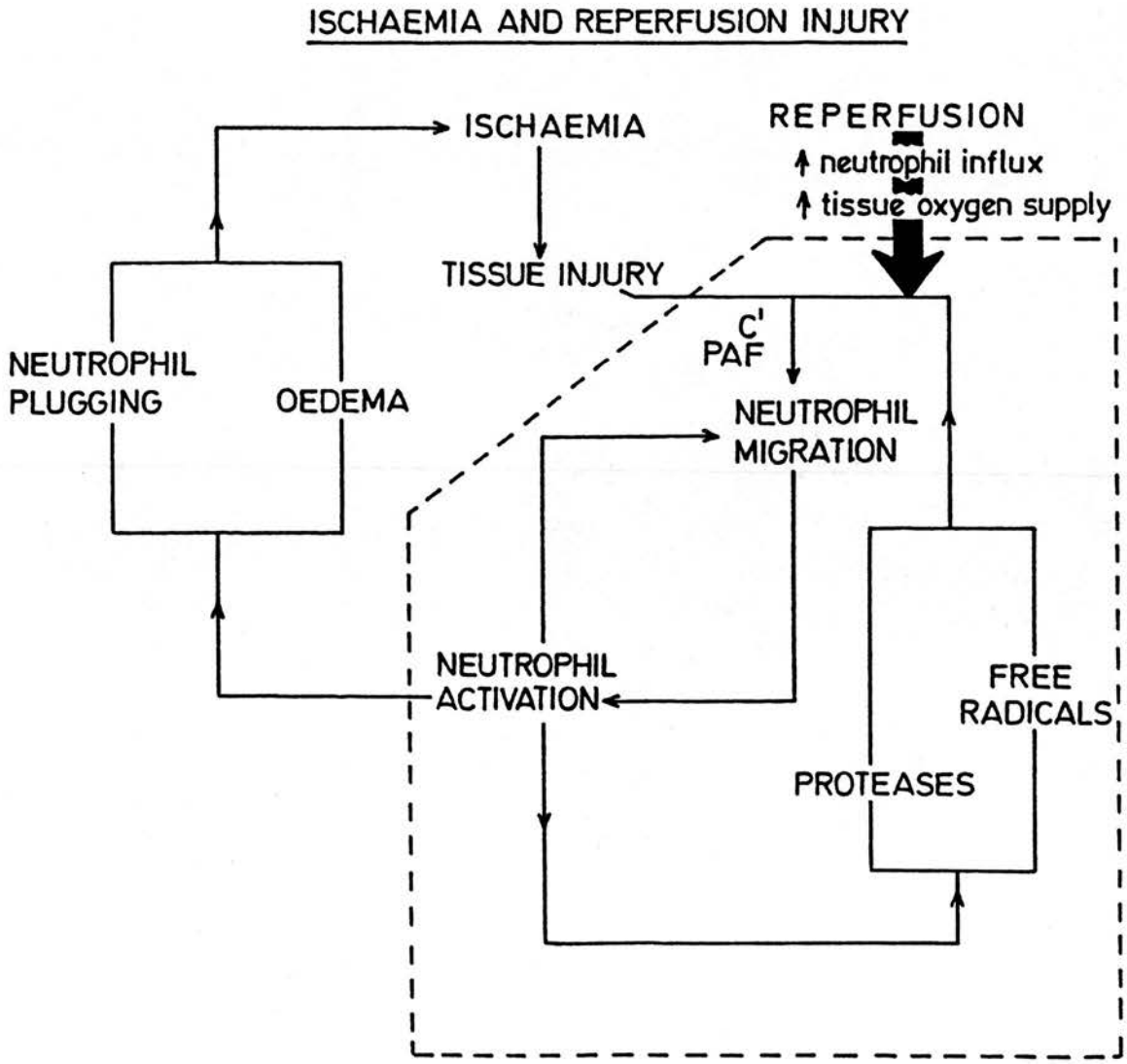


Fig 2d Diagram showing a potential role for the neutrophil and the acute inflammatory response in reperfusion injury.

phenomenon probably represents an extension of the normal cellular response to tissue injury and is now well described in the heart (Kloner, 1974; Engler, 1986). A substantial body of evidence now exists which indicates that reactive oxygen species are involved in the microvascular and parenchymal tissue injury associated with reperfusion of ischaemic tissues (McCord, 1985). In addition to the neutrophil other sources of free radical production, following myocardial ischaemia, include the conversion of xanthine dehydrogenase to xanthine oxidase, mitochondrial production, the auto-oxidation of catecholamines and the arachidonic acid cascade (Werns, 1986). Of these mechanisms, the xanthine dehydrogenase pathway is well studied and may contribute to endothelial cell damage but is unlikely to damage myocardium as this enzyme is not present within myocytes (Manfredi, 1985). Irrespective of the source of free radicals experimental models have shown that free radical scavengers and agents which prevent free radical production provide significant myocardial protection in myocardial ischaemia followed by subsequent reperfusion (Schlafer, 1982; Ambrosio, 1986).

The third mechanism by which neutrophils may produce myocardial damage following reperfusion is by the release of potent proteolytic enzymes. Neutrophil elastase is known to be implicated in endothelial damage in vitro (Smedley, 1986) but there has been little study of this in

vivo and no study in relation to myocardial injury.

There is increasing experimental evidence which implicates the neutrophil in secondary myocardial injury following myocardial infarction and in reperfusion injury. There has, however, been little work examining the role of the acute inflammatory response in myocardial injury in man. The object of this thesis was to study this question in man, particularly as it may have therapeutic benefits if neutrophil mediated secondary myocardial injury contributes to secondary myocardial damage following infarction.

Neutrophil behaviour following myocardial infarction was studied in three ways; using radiolabelled autologous neutrophils to image the neutrophil infiltrate at the site of myocardial damage, by measuring plasma neutrophil elastase as a marker of neutrophil activation and by measuring the non-peroxide diene conjugate of linoleic acid as a marker of free radical activity.

The methods for neutrophil separation and radiolabelling are described in Chapter 3 as well as the subsequent in vivo behaviour of the radiolabelled cells. The factors influencing the uptake of Indium-111 (^{111}In) labelled neutrophils at the site of myocardial damage are discussed in Chapter 4. In Chapter 5 a method for measuring

infarct size (using Technetium-99m pyrophosphate) and evaluating the extent of ^{111}In-labelled neutrophil infiltrate is described, using single photon emission computed tomography. The effect of treatment with and without thrombolytic therapy on these parameters is also studied.

In Chapter 6 the method for measuring plasma neutrophil elastase, as a marker of neutrophil activation in normal control subjects, patients with ischaemic heart disease and acute myocardial infarction is described. The method for measuring the non-peroxide diene conjugate of linoleic acid, as a marker of free radical activity in the same groups of subjects is described in Chapter 7. In acute myocardial infarction the patterns of appearance of elastase and the diene conjugate were followed in the first 48 hours following the onset of symptoms and the effect of treatment with thrombolytic agents on these patterns also studied in Chapters 6 and 7. Chapter 8 summarises the findings and future avenues of research are discussed in Chapter 9.

CHAPTER 3

THE SEPARATION, RADIOLABELLING AND SUBSEQUENT IN-VIVO BEHAVIOUR OF AUTOLOGOUS NEUTROPHILS

INTRODUCTION

The potential use of radiolabelled blood cell elements for non-invasive diagnosis and improving the knowledge of in-vivo cell behaviour has long been recognised. Initial progress was thwarted, however, by the lack of a radionuclide to label blood cells which was also suitable for gamma camera imaging. In the last decade it has been shown that leucocytes, and other cell elements, can be labelled efficiently with Indium-111(^{111}In) chelated with 8-hydroxyquinolone (oxine) while retaining their in vitro and in vivo functional activity (Thakur, 1977; Zakhireh, 1979). ^{111}In-oxine is lipid soluble and diffuses passively through the cell membrane thereby allowing efficient incorporation of radioactivity into isolated blood cells (Thakur, 1977b). The half life of ^{111}In of 67 hours makes it suitable for cell kinetic studies as well as determining subsequent localisation of the radiolabelled cells without excessive radioactive decay, while the two gamma photons (173 keV and 247 keV) permit adequate gamma camera imaging.

^{111}In-labelled autologous neutrophils are often used diagnostically to detect occult infection, but can also be used to demonstrate active inflammation as in rheumatoid arthritis (Uno, 1986), pancreatitis (Andersen, 1986) and inflammatory bowel disease (Saverymuttu, 1982). In much of the initial work mixed cell populations were labelled but there has been increasing concern about the labelling of radiosensitive cells, such as the lymphocytes, as ^{111}In-oxine may induce severe chromosomal aberrations (ten Berge, 1983). Further the use of mixed cell population will result in labelling of cells not directly involved in the inflammatory response, particularly erythrocytes, which leads to poorer imaging with possible false positive results because of blood pool activity. In addition, mixed cell populations do not permit accurate assessment of neutrophil responses and in-vivo behaviour in disease states. For this reason the separation and radiolabelling of pure neutrophil preparations is now recommended (Thakur, 1981).

A number of techniques for the isolation and labelling of pure neutrophils exist, but in general they are time consuming and involve a number of steps including an initial red cell sedimentation step with subsequent preparation of discontinuous density gradients (Boyum, 1968). Percoll and Ficoll-Hypaque are two density gradient media commonly used for the isolation of

neutrophils. Percoll is based on colloidal silica coated with polyvinylpyrolidone while Ficoll-Hypaque is a mixture of a polysaccharide and a radiopaque contrast media. It has been suggested that Percoll produces less in vitro cell activation than Ficoll-Hypaque but this may have been due to contamination of the Ficoll-Hypaque by bacterial lipopolysaccharide (Haslett, 1985). There is also debate about the best method of radiolabelling cells with ^{111}In (Danpure, 1982; Goedemans, 1982), but of the currently used labels ^{111}In-oxine is the most studied. Despite the debate as to the best method, most workers agree whichever method is used it is important that radiolabelled neutrophils show normal in vivo kinetics in terms of transit through lungs, and localisation in liver and spleen (Saverymuttu, 1983). Non-viable cells show greater lung retention with increased liver sequestration (Thakur, 1977; Saverymuttu, 1983).

As the main purpose of this thesis was to examine the role of the acute inflammatory response in myocardial infarction, an acute medical emergency, a technique which was rapid and simple to use was required to allow neutrophil labelling soon after the patient's admission. Mono-poly resolving medium (MPRM) is a commercially available mixture of Ficoll-Hypaque which had been used to isolate neutrophils for in vitro work from 5 ml of whole blood in a single step (Ferrante, 1978). It had not been

used for the preparation of autologous labelled neutrophils for in vivo studies. Equally, 5 ml of blood would provide insufficient neutrophil recovery for subsequent labelling and imaging, therefore this technique was evaluated as a method for isolating neutrophils in a single step from 50 ml of whole blood. In addition to measuring neutrophil recovery and efficiency of cell labelling the transit of the radiolabelled neutrophils through lung, liver and spleen was assessed after intravenous bolus injection. Evidence of active migration to sites of infection or inflammation was also sought by subsequent whole body gamma camera imaging of the patients.

PATIENTS AND METHODS

Patients

Investigations with ^{111}In-labelled autologous neutrophils were performed on 50 patients with suspected infection, and various inflammatory conditions including acute myocardial infarction (table 3 (i)). In 8 of these subjects with no history of smoking or clinical or radiological evidence of cardiopulmonary disease, the pulmonary and blood pool clearance of ^{111}In-labelled neutrophils was assessed at the same time as accumulation in liver and spleen.

Cell separation

Venous blood was collected into a 60 ml syringe, containing 300 units of preservative free heparin (Leo Laboratories, Buckinghamshire, UK) via a 19G infusion set. A full blood count and ESR were performed on 10 ml of blood. All subsequent procedures were performed using aseptic technique. In duplicate, 25 ml of blood was layered over 12 ml Mono-poly Resolving Medium (Flow Laboratories Ltd, Irvine, Ayrshire, UK) in a sterile tube and centrifuged at 400G for 60 minutes. Differential migration during centrifugation results in two cell bands, one containing mononuclear cells and the other containing polymorphonuclear cells, with a red cell pellet (fig 3a). From the top plasma layer 8 ml was collected and centrifuged at 1000g for 10 minutes to provide platelet poor plasma (PPP). The remaining plasma and the upper cell band containing the mononuclear cells was discarded. The neutrophils were then recovered from the lower cell band, resuspended in 40 ml of phosphate buffered saline (PBS, pH 7.4) and centrifuged at 400g for 10 minutes. The supernatant was discarded and the cell pellet resuspended in 10 ml of PBS. A 1 ml sample was taken to determine total and differential white cell counts. Red cell contamination was assessed in the 8 subjects in whom first pass studies were performed.

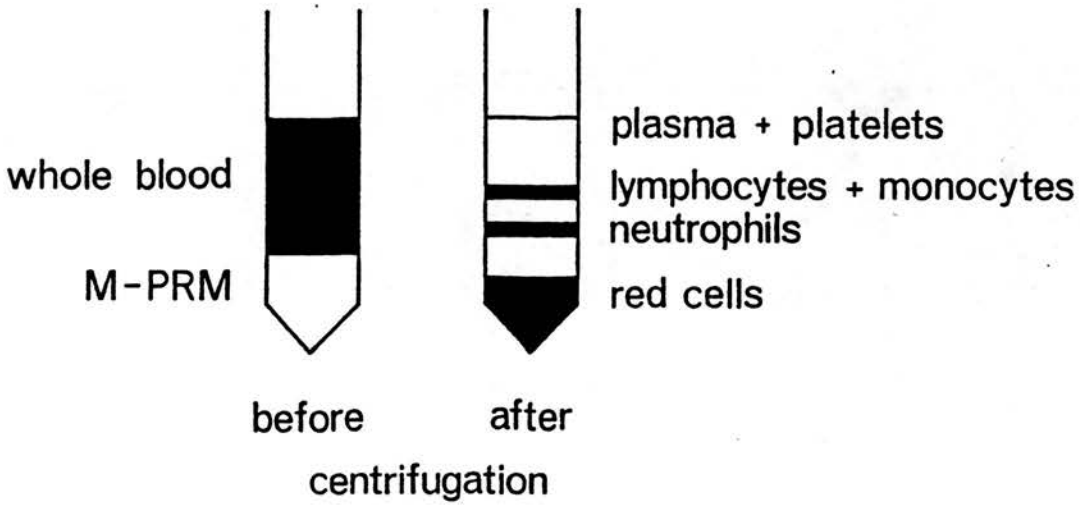


Fig 3a Diagrammatic representation of the separation of whole blood using Mono-poly resolving medium (MPRM). After centrifugation three distinct cell bands are seen, the neutrophils can be harvested from the middle band.

Labelling procedure

^{111}In-oxine solution (20-49 MBq) (Amersham International plc, Amersham, UK) was added dropwise to the remaining suspension of neutrophils. After incubation at room temperature for 15 minutes, 3 ml of PPP was added, then the cell suspension centrifuged at 250g for 10 minutes. The supernatant was transferred to a fresh tube to count the amount of unlabelled ^{111}In. The cell pellet was then resuspended to a volume of 5 ml with equal parts of PBS and PPP. The activities of the cell suspension and supernatant were then measured in a radioisotope calibrator (ARC-120, Capintex Inc, New Jersey, USA) and the labelling efficiency calculated. The cell suspension was then drawn into a syringe ready for injection.

Cell counting

Cell counts were performed manually using a new improved Neubauer chamber (0.100 mm). Blood films made from whole blood and neutrophil suspension were stained with May-Grunwald/Giemsa and a differential cell count determined. The percentage cell recovery was calculated by comparing the neutrophil count in whole blood with that in the neutrophil suspension.

Gamma camera imaging and kinetic studies

Imaging was performed using a large field of view gamma camera (GEC-400 AT, Maxicamera) with a medium energy

collimator and energy windows set at 155-195 keV and 220-285 keV. The gamma camera was linked to a PDP 11-34 computer (Digital Equipment Corporation).

Static planar images of the chest, abdomen and pelvis were obtained in all subjects at 24 hours after injection.

Kinetic data were obtained in 8 subjects in the following manner. The subject was positioned with the gamma camera anteriorly over the chest and upper abdomen to allow subsequent visualisation of lung, liver, heart and spleen. A 19G venflon was then inserted into the right antecubital fossa and attached to a 5% dextrose infusion. The 5 ml of ^{111}In-labelled autologous neutrophils was then injected as a bolus followed by a fast running 5% dextrose infusion. A sequence of 64 x 64 matrix images was acquired, from the moment of injection, over a 25 minute time period with a varying time frame interval starting at 5 seconds and then increasing stepwise to 60 seconds for the later images. The counts were then expressed as counts per second to normalise for the varying frame length.

To assess ^{111}In-neutrophil kinetics, the computer derived planar images were inspected and regions of interest (ROI) drawn manually around the heart, lungs, liver, spleen and the whole field of view (fig 3b). The whole field plane

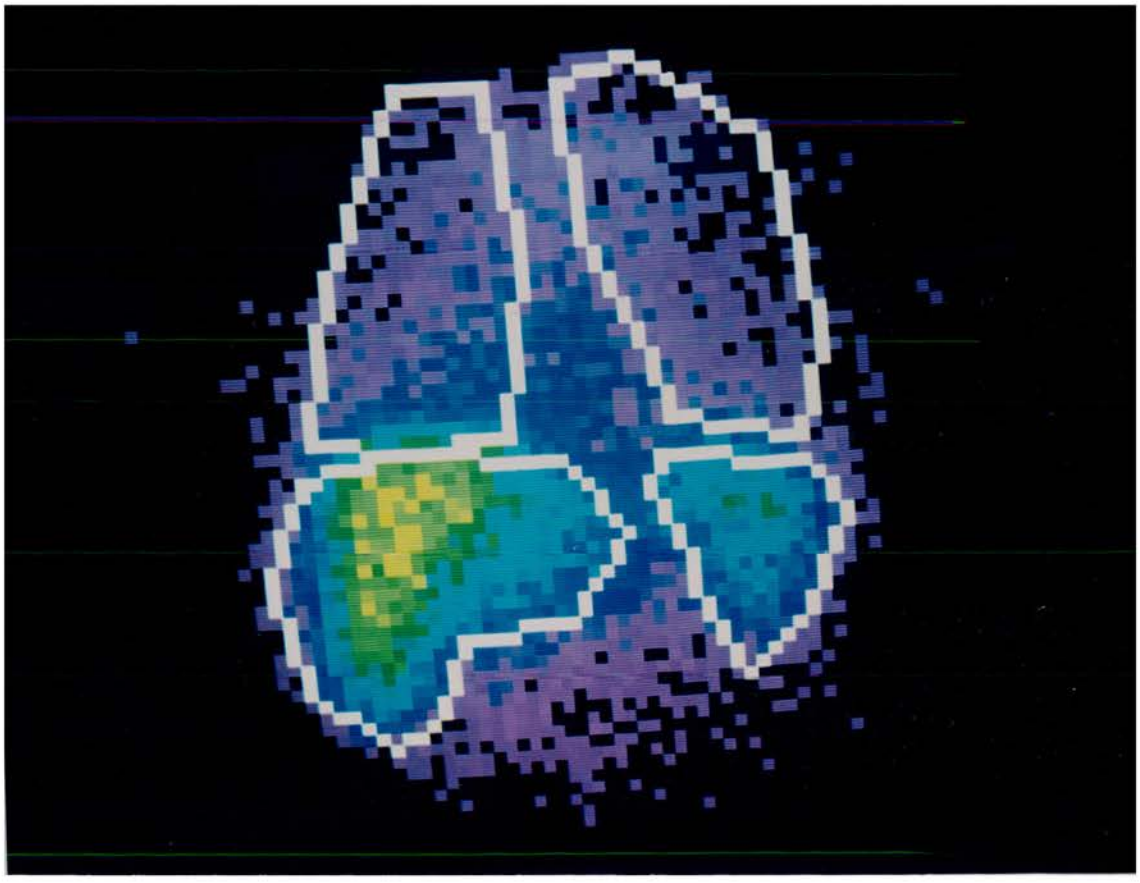


Fig 3b Computer image approximately 15 minutes after injection of ^{111}In-labelled neutrophils showing the regions of interest drawn around lung, liver and spleen. Few neutrophils remain within the pulmonary circulation at this stage.

showing the maximum count-rate was assumed to represent the total activity injected and was always in the first 1-3 frames. The count rates from the other ROI's were then expressed as a percentage of the maximum whole field count rate and a time-activity curve created for each organ.

RESULTS

All results are expressed as mean ± SD.

Neutrophil recovery and labelling efficiency

Individual patient results are given in table 3(i). In 5 of the 50 patients studied, there was no clearly detectable neutrophil band and therefore these were excluded from further analysis. From the remaining 45 blood samples, the neutrophil recovery was 50.3 ± 17.8% and only one sample contained the minimum recommended number of cells for labelling of 1.5×10^7. All neutrophil suspensions were labelled with ^{111}In-oxine with a mean labelling efficiency of 70.8 ± 13.2%. In the 8 subjects in whom kinetic studies were performed red cell contamination was 5.7 ± 2.6% and lymphocyte contamination less than 0.5%.

Kinetic studies

The individual time activity curves for the 8 patients are listed in table 3 (ii). The data for each organ are

expressed as percentages of the maximum count rate achieved in the whole field of view.

Heart count rates, which relate to circulatory blood pool activity, for the group fell to 3% within 90 seconds and after 5 minutes remained constant at 2% for the remainder of the study. Lung count rates fell to 19% within the first 90 seconds and thereafter fell slowly to 9% by 20 minutes. The liver counts rose rapidly to 16% by 15 minutes thereafter remaining constant, whereas the count rate over the spleen continued to rise throughout the study. The mean data for the 8 subjects is shown in figure 3 (c).

Imaging results

Of the 45 patients in whom cell separation and labelling was performed, all underwent planar imaging. In 18 patients positive images were obtained; 9 patients with acute myocardial infarction, 4 localised abscess collections, 2 patients with colitis, 1 patient with pneumonia, 1 with Mycotic aneurysm, 1 with infected venous access site. In none of the patients with suspected occult infection, who had negative ^{111}In-autologous neutrophil scans, was this subsequently detected. Examples of positive scans are given in figures 3d and 3e.

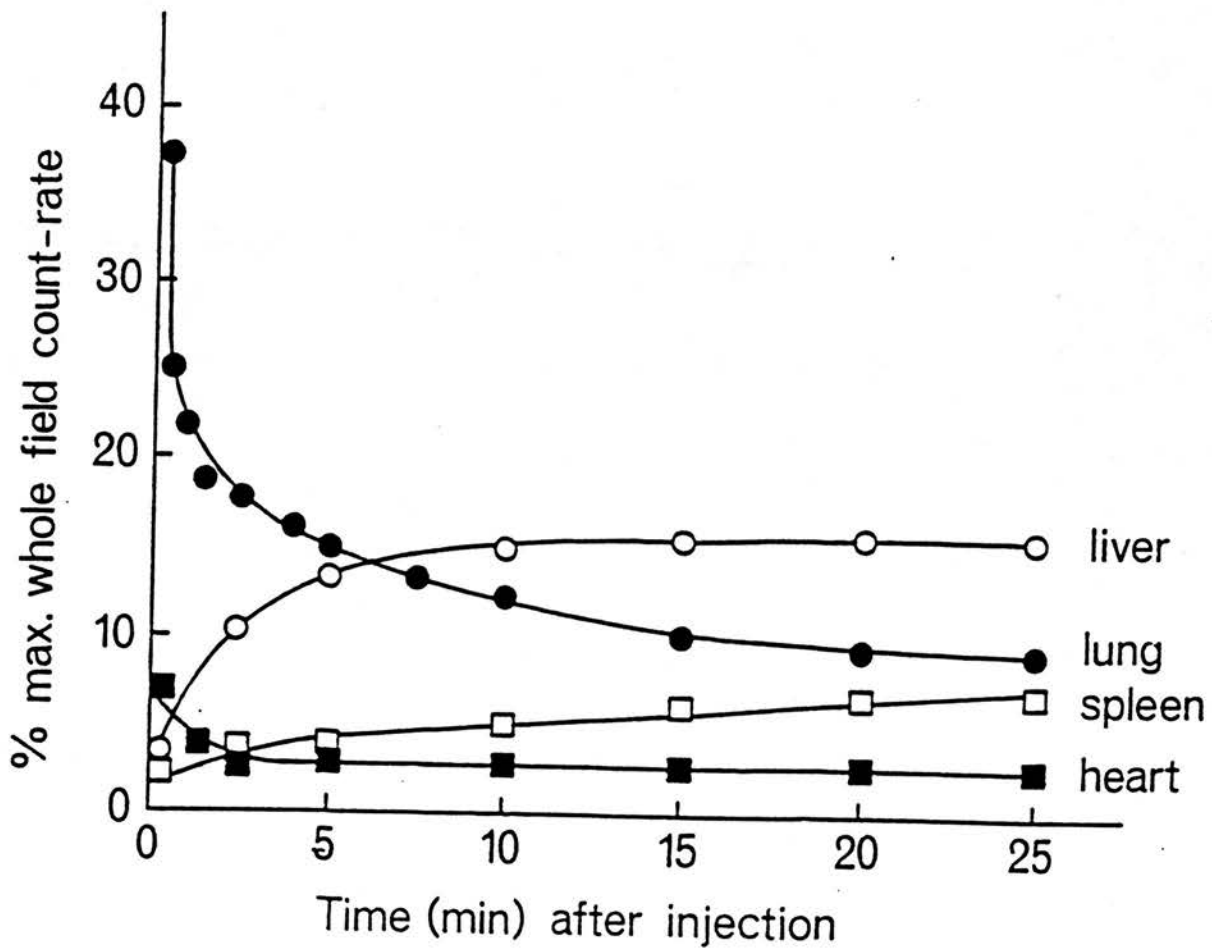


Fig 3c Mean data showing the ^{111}In-labelled neutrophil time courses for lungs, heart, liver and spleen.

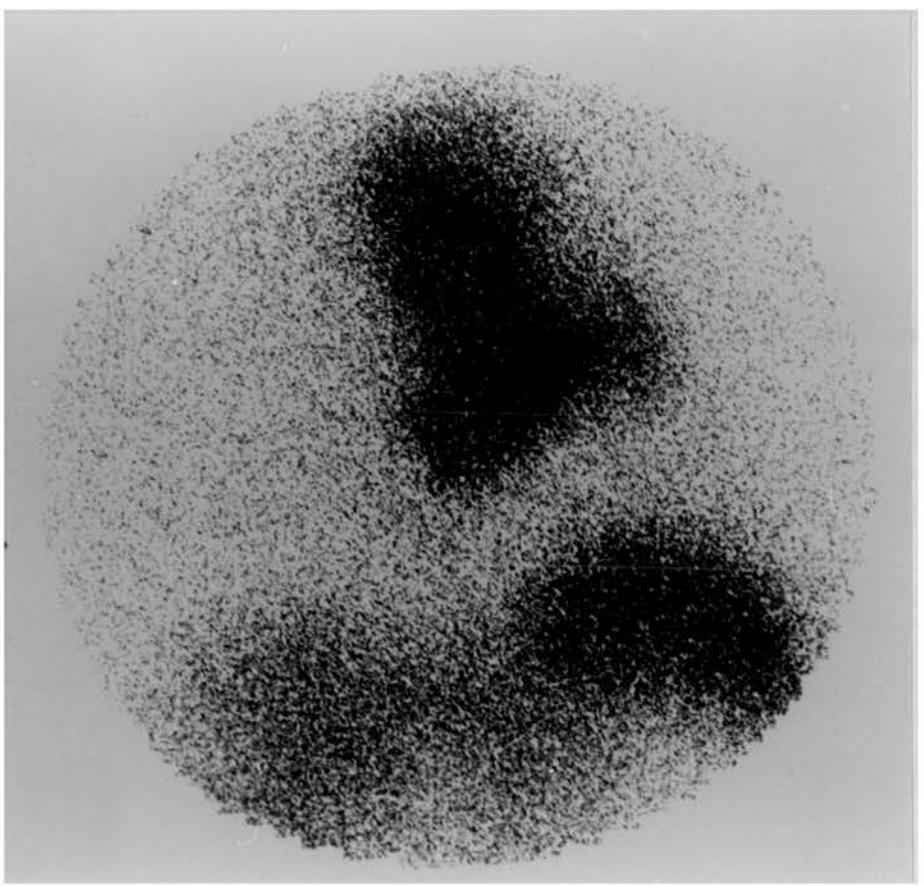


Fig 3d Anterior planar image in a patient with an extensive left upper lobe pneumonia. Note abnormal uptake of ^{111}In-neutrophils in the left lung with normal uptake in liver and spleen.

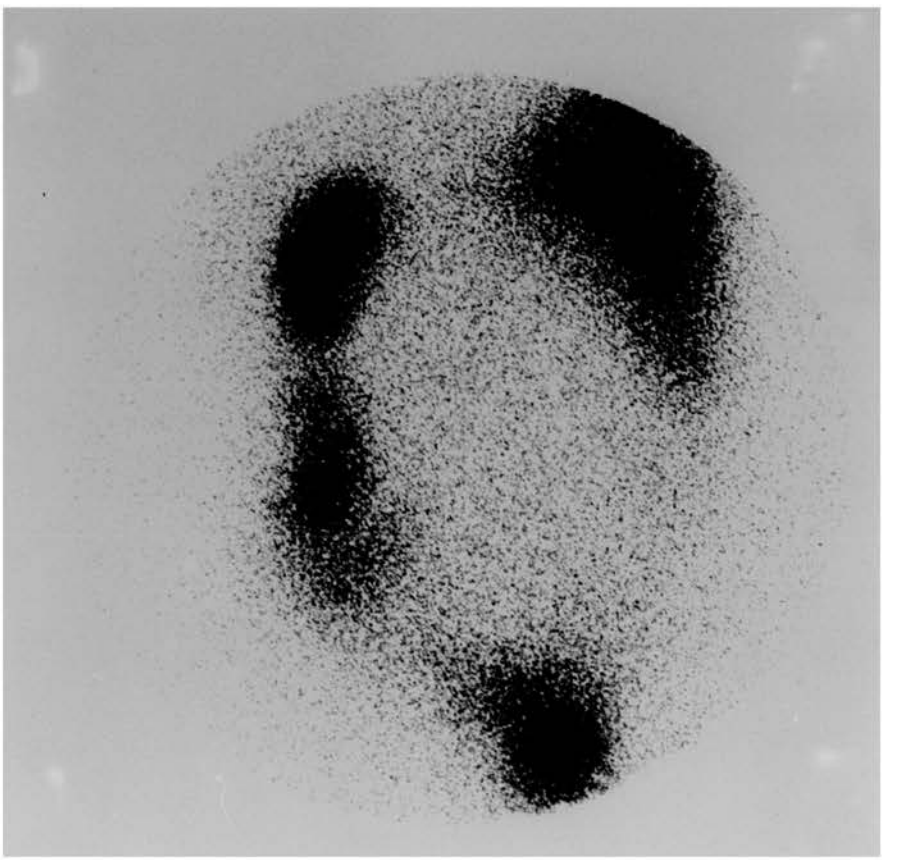


Fig 3e Posterior planar image in a patient with ischaemic colitis of the descending colon. note abnormal uptake of ^{111}In-neutrophils in colon from the splenic flexure to the rectum with normal uptake in liver and spleen.

DISCUSSION

The results show that the described single step technique with MPRM can be used to reliably separate neutrophils in sufficient numbers for subsequent labelling. The method has a number of advantages. Firstly, it avoids an initial red cell sedimentation step with dextran, hydroxyethyl starch or methylcellulose as is currently normal practice (Danpure, 1982; Peters, 1983; Thakur, 1977a). Secondly, the method described does not require the preparation of a discontinuous density gradient with Percoll/saline of Percoll/plasma (Saverymuttu, 1983) and is therefore technically less demanding. The third major advantage is that the method is more rapid than those previously described for "pure" neutrophil preparations and involves less handling of the cells with the concomitant risk of mechanical cell damage. Of the 50 attempted separations 5 were failures of which 3 patients had documented chronic obstructive lung disease. We have subsequently shown that this method is unreliable when attempting to separate neutrophils from whole blood in this group of patients and this may relate to morphological or functional changes induced in red or white cells by smoking. In the remaining patients in whom clear neutrophil bands were seen, neutrophil recovery was excellent and the cell yield more than sufficient for good radiolabelling. Labelling efficiency is known to improve

as neutrophil concentration increases, a factor which is of importance if there is red cell contamination (Danpure, 1982). The extent of red cell contamination was small, comparing favourably with previously published results (Saverymuttu, 1983), and unlikely to interfere with the radiolabelling of neutrophils with ^{111}In-oxine as neutrophils are labelled more efficiently than red cells (Danpure, 1982). Overall the labelling efficiency was good at 70% and in agreement with previous studies (Thakur, 1977).

A number of studies have shown that poorly functioning or damaged radiolabelled neutrophils will show prolonged lung retention or greater liver sequestration (Thakur, 1977; Weiblen, 1979; Saverymuttu, 1983). It was therefore important to demonstrate that the cells separated with MPR-M and subsequently labelled with ^{111}In-oxine demonstrated normal in-vivo kinetics. Our results are consistent with functioning neutrophils with rapid clearance from the pulmonary circulation, 80% of the original activity clearing in 90 seconds and although the magnitude of change is different, the ^{111}In-time-activity curves are similar for both heart and lung. The slight difference in the two slopes is consistent with a degree of lung retention, but this probably represents the normal marginated pool of neutrophils in the pulmonary circulation (Ambrus, 1954). Further, the difference in

the curves at 25 minutes, lung counts being greater than the heart counts, may also partly be explained by the greater blood volume in the pulmonary vasculature compared to heart. Splenic uptake continues to increase over the study period compatible with pooling of the neutrophils in the splanchnic vascular bed, whereas the count rates over the liver plateau after approximately 15 minutes which is against continued removal of damaged cells by the liver. These data are consistent with previously described neutrophil kinetic studies, and confirm normal in vivo behaviour of the cells separated and radiolabelled by this method.

Further, these radiolabelled neutrophils have been shown to migrate to sites of inflammation or infection which implies that they are capable of responding to normal inflammatory stimuli. The results also suggest that the acute inflammatory response to acute myocardial infarction can be imaged by this technique, but this was not invariable and the factors which influenced this are analysed further in Chapter 4.

CHAPTER 4

IMAGING THE INFLAMMATORY RESPONSE TO ACUTE MYOCARDIAL INFARCTION USING INDIUM-111 LABELLED AUTOLOGOUS NEUTROPHILS

INTRODUCTION

With the development of large crystal scintillation cameras (Anger, 1963) and improved radiopharmaceuticals, radionuclide techniques for cardiac imaging have become part of routine clinical practice. The imaging agents which are commonly used are Technetium-99m (^{99m}Tc) labelled pyrophosphate for infarct avid imaging, ^{99m}Tc labelled human serum albumin or red blood cells for blood pool and radionuclide ventriculography, and Thallium 201 for studying regional alterations in myocardial perfusion. A further recent advance in myocardial imaging has been the introduction of single-photon emission computed tomography (SPECT) (Holman, 1979). This has become possible through new developments in computing combined with the introduction of a rotating gamma camera which has allowed three dimensional images to be reconstructed. The major benefits of SPECT for cardiac imaging are improved spatial separation of radioisotope uptake in skeleton or chest wall from myocardium, plus the ability to quantify individual tomograms allowing accurate

measurement of infarct size or the area of ischaemia (Jansen, 1985).

In addition to diagnostic imaging, radiolabelled blood cells have also been used for myocardial imaging in experimental models. Radiolabelled platelets have been used to image acute coronary thrombosis and infective endocarditis (Riba, 1979), while mixed leucocyte preparations were first used to image the inflammatory response to myocardial infarction in dogs. Further studies using ^{111}In-labelled autologous neutrophils showed that anatomically distinct areas of increased myocardial radioactivity uptake could be detected in all dogs studied within 24-96 hours after infarction (Thakur, 1979). A study in man evaluating the use of ^{111}In-labelled white cell imaging, however, failed to detect any abnormal activity in three patients following acute myocardial infarction (McDougall, 1979). The feasibility of imaging the inflammatory response to acute myocardial infarction was better assessed in a later study which found that positive uptake of labelled white cells could be seen in over 50% of patients studied (Davies, 1981). The only factor which appeared to influence the uptake of radiolabelled neutrophils was the time from onset of chest pain to reinjection of the cells; all patients injected within 24 hours subsequently had positive images.

The object of this part of the study was to determine whether the method previously described in Chapter 3 to separate and label autologous neutrophils allowed reliable imaging of the acute myocardial infarction in man. Factors which could potentially influence uptake of neutrophils at the site of myocardial damage were also studied including time from onset of symptoms to injection, infarct size and concomitant drug therapy.

Single photon emission computed tomography was also combined with conventional planar imaging to assess whether this improved localisation and detection of myocardial uptake of radiolabelled neutrophils.

PATIENTS AND METHODS

Patients

Thirty patients with acute myocardial infarction admitted to the Coronary Care Unit were studied. All gave a history of prolonged ischaemic chest pain, lasting longer than 30 minutes, with electrocardiographic changes associated with myocardial infarction and subsequently had a rise in serum creatine kinase at least twice the upper limit of normal. Infarct location was characterised according to established electrocardiographic criteria as anterior (including anteroseptal and anterolateral) or inferior (including inferolateral and inferoposterior).

Patient details including time of reinjection of ^{111}In-labelled neutrophils, activity administered and full drug history are documented in table 4 (i).

METHODS

Preparation of ^{111}In-labelled neutrophils

Autologous neutrophils were separated from 60 ml of whole venous blood and labelled with ^{111}In-oxine as described in the methods section of Chapter 3.

Patient imaging

Within two and a half hours of the initial venesection, all patients were reinjected with resuspended ^{111}In-labelled autologous neutrophils (5 ml). Cardiac imaging was performed the following day approximately 24 hours after reinjection, therefore the earliest imaging time for any patient was 36 hours after the onset of chest pain and the latest 57 hours. The study protocol is shown in fig 4a.

All patients were imaged supine and standard planar images acquired in the anterior, left anterior oblique and left lateral position for 100,000 counts using a gamma camera (GEC-400AT-Maxicamera). In 24 patients SPECT was performed using the same gamma camera linked to a DEC PPP11/23 computer using locally written software. Ten

PROTOCOL

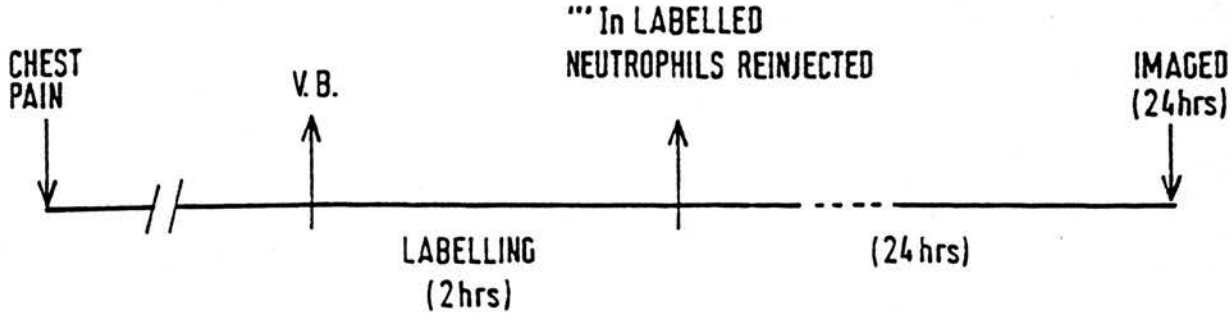


Fig 4a Study protocol. All times are related to the onset of symptoms and patients were imaged approximately 24 hours after reinjection of the radiolabelled autologous neutrophils.

minutes prior to the SPECT study, 40 MBq of ^{99m}Tc labelled human serum albumin was administered to allow blood pool imaging and hence myocardial localisation. A sequence of 64 simultaneous two dimensional images was acquired for both ^{111}In and ^{99m}Tc as the head of the gamma camera rotated through 360°, starting in the right anterior oblique position (fig 4b). The total imaging time was 32 minutes and at the end of this period all 64 projections are summed independently for both radionuclides, and computer reconstructed to provide transverse, sagittal or coronal images (fig 4c).

Image interpretation

The images for planar and SPECT imaging were assessed separately. Planar imaging was graded positive when Indium activity was clearly seen in the region of the heart and negative where there was no detectable activity or if the area of activity was inseparable from liver, spleen or skeletal uptake. Dual isotope single photon emission computed tomographic images were considered to be positive when Indium activity was seen in all three reconstructed views in relation to the ^{99m} Tc blood pool image.

Statistical analysis

Standard statistical methods were used to calculate means and standard deviation. Results from the two different

Fig 4b

Single photon emission computed tomography of the myocardium is performed with the patient lying supine while the gamma camera rotates through 360^{o} starting from the right anterior oblique position.

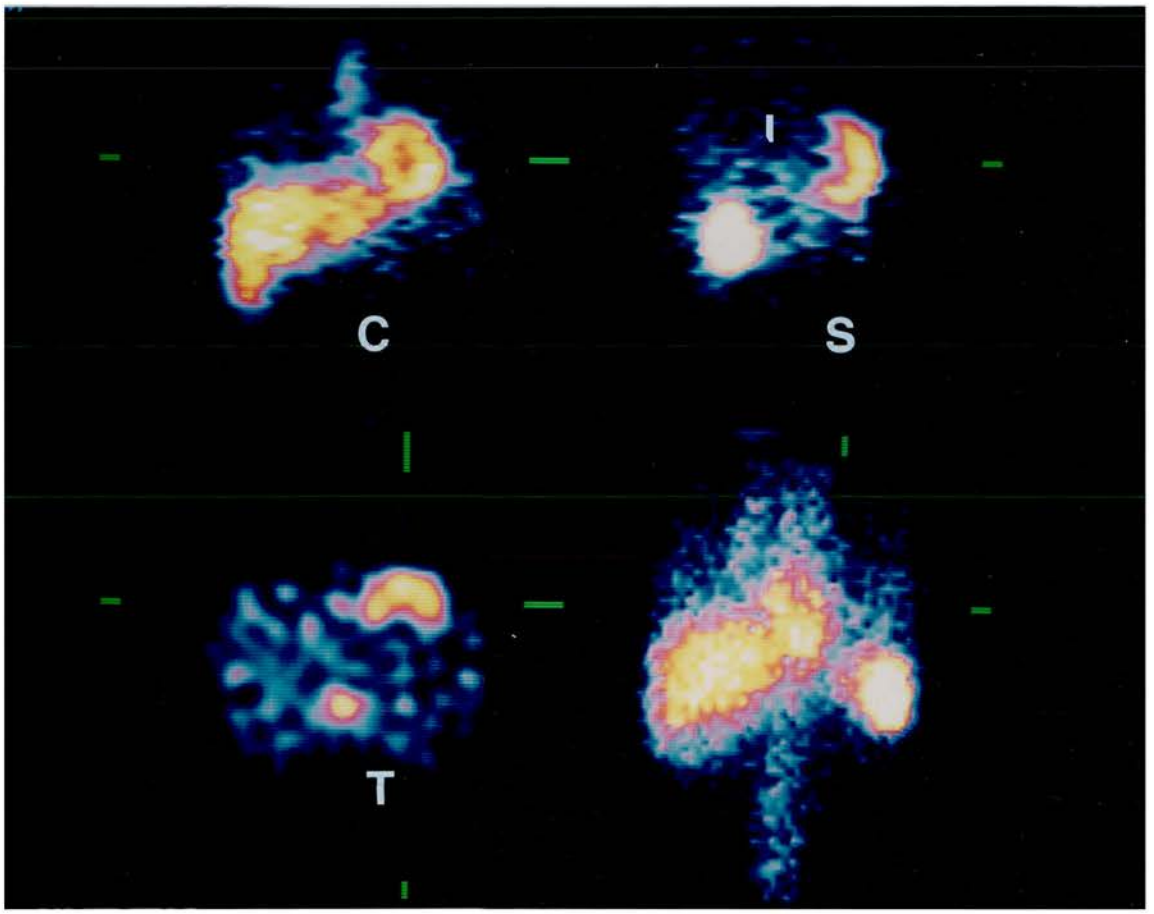


Fig 4c Computer reconstructed images showing uptake in the transverse (T), sagittal (S), coronal (C) planes in a patient with anterior myocardial infarction using ^{111}In-labelled neutrophils. "3D" image also shown.

populations, those with positive and negative images, are compared using Student's unpaired 't' test or exact probability test or unpaired Wilcoxon rank sum test as appropriate. Values of $p > 0.05$ were not considered significant.

RESULTS

Of the 30 patients with acute myocardial infarction, 23 had positive uptake of ^{111}In-labelled neutrophils within the myocardium using a combination of planar imaging and SPECT. Three patterns of uptake were seen on planar imaging: focal myocardial uptake (12 patients), diffuse myocardial uptake (3 patients) and "doughnut pattern" (2 patients). Figure 4d shows an example of diffuse and focal uptake and figure 4e a doughnut pattern of uptake.

In 6 patients where the planar images were considered to be negative or equivocal, SPECT reconstruction showed localised uptake within the myocardium an example is given in fig 4f. In addition, the use of dual isotope SPECT improved anatomical localisation of the infarct by confirming its relation to cardiac blood pool imaged with ^{99m}Tc-labelled human serum albumin (fig 4g).

The details of the 23 patients with positive images and 7 patients with negative images are summarised in table 4

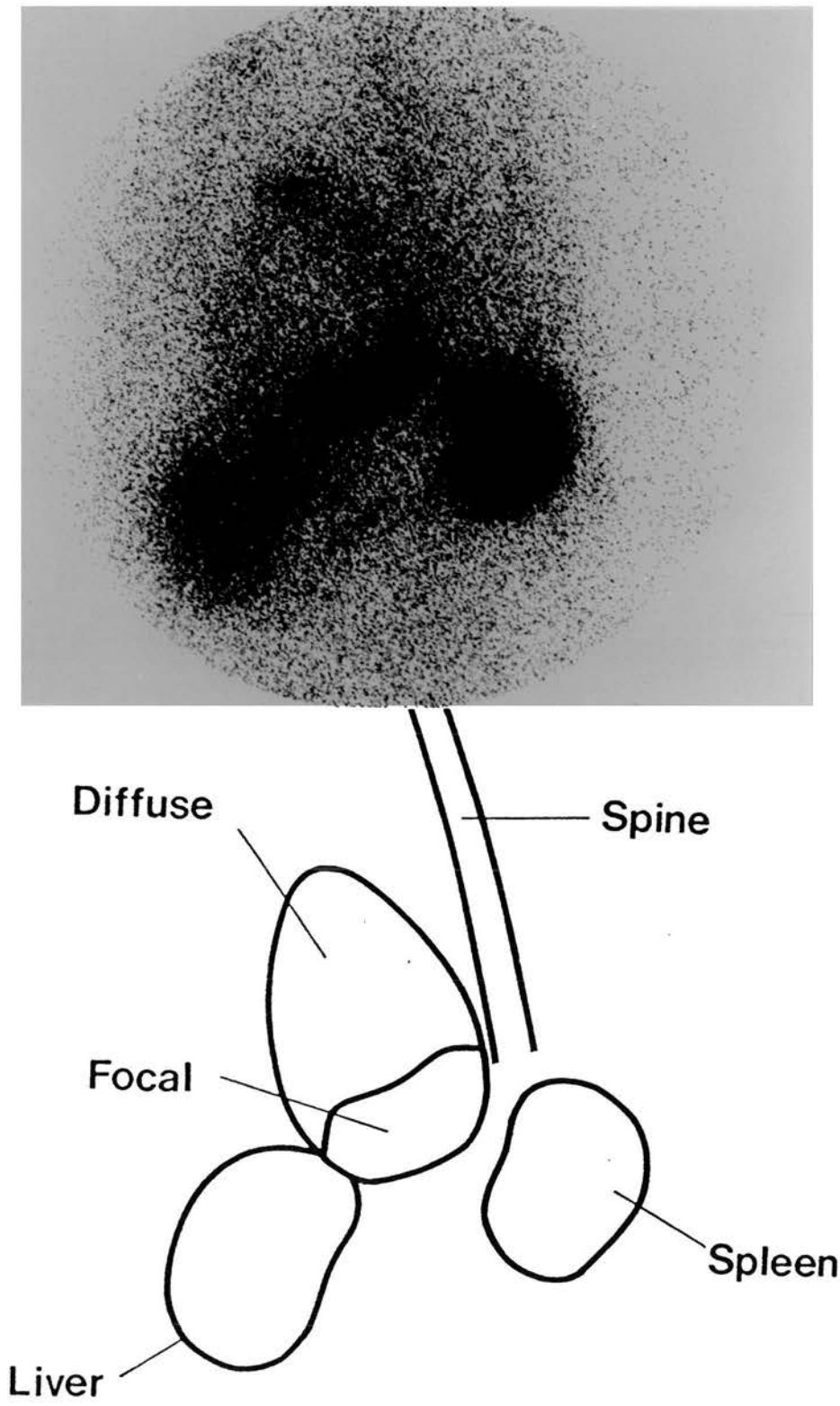


Fig 4d

Planar image in the left anterior oblique view in a patient with acute myocardial infarction using ^{111}In-labelled neutrophils showing focal and diffuse uptake within the myocardium; note normal uptake in liver and spleen. The line drawing shows the areas of uptake.

Fig 4e Planar image in the anterior view in a patient with acute myocardial infarction showing a "doughnut pattern" of uptake within the myocardium of ^{111}In-labelled neutrophils.

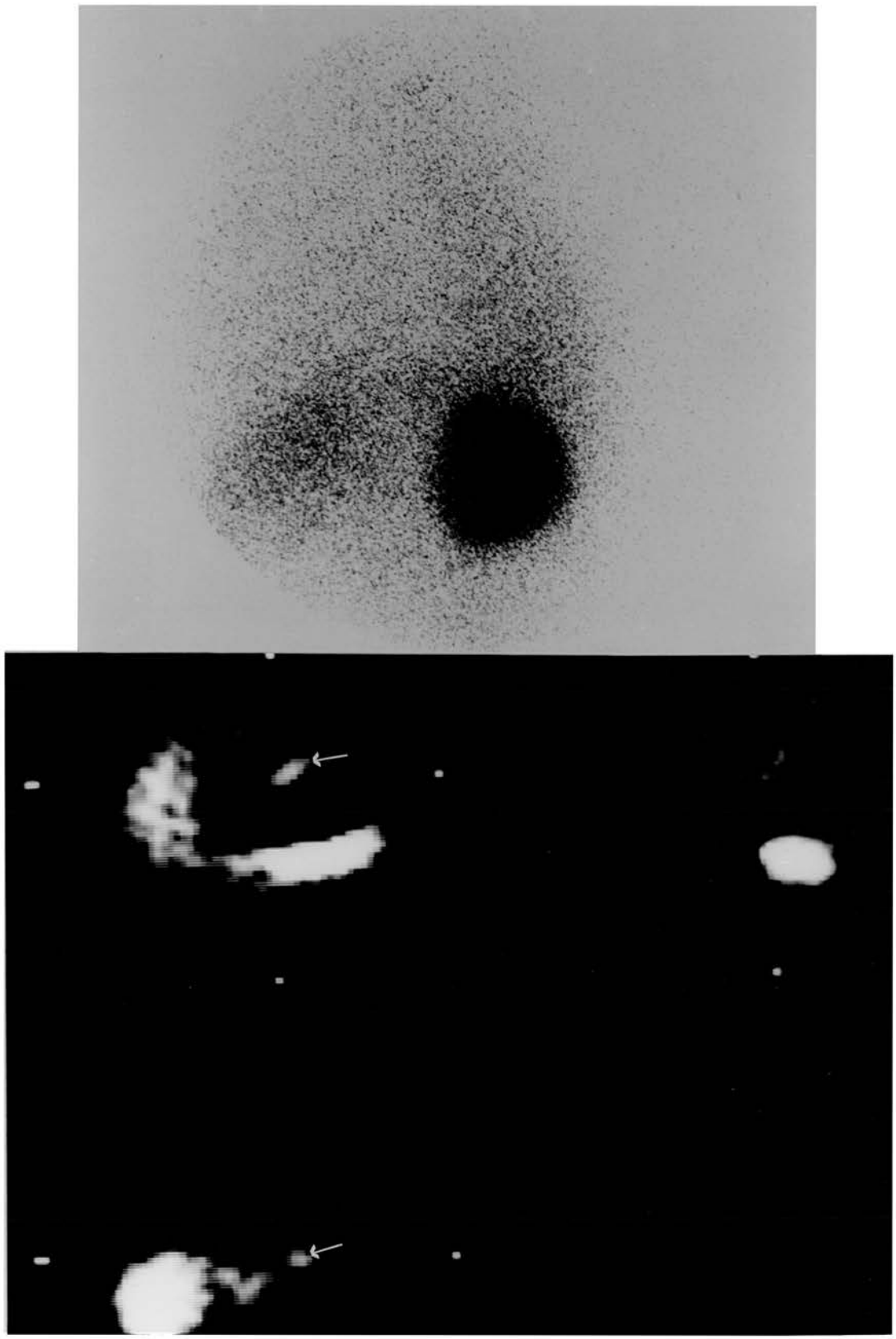


Fig 4f Planar image in the left lateral view in a patient with myocardial infarction in whom no definite uptake of radiolabelled neutrophils can be seen (upper). The corresponding SPECT reconstruction images (lower) show definite myocardial uptake in all three views (arrowed).

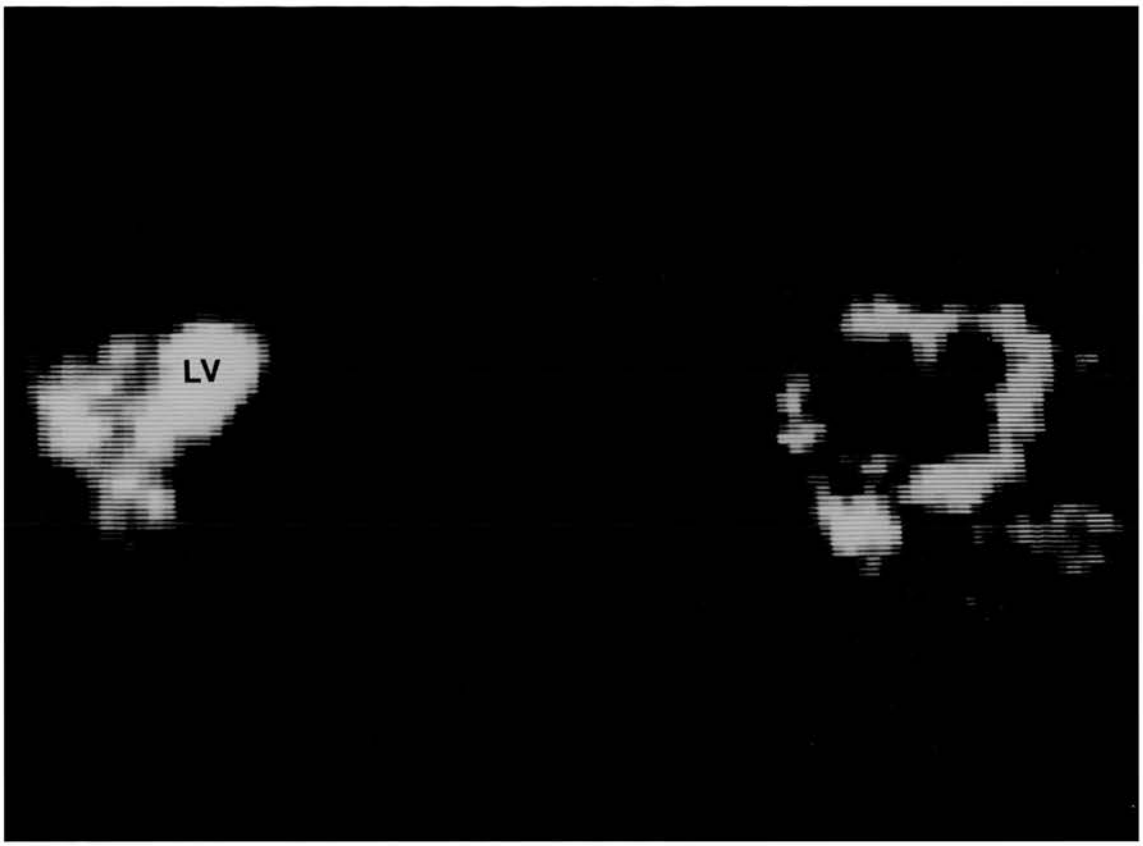


Fig 4g Simultaneous single photon emission computed tomographic images in the transverse plane. The ^{99m}Tc image shows blood pool (left) in the left and right ventricles while the ^{111}In image (right) shows extensive uptake within the myocardium of both ventricles.

(i). The percentage of positive images increased as the interval between onset of symptoms and injection of ^{111}In-labelled neutrophils shortened; all patients injected within 18 hours of infarction had positive images, whereas only 4 out of 9 patients had positive images when injected after 24 hours (fig 4h). Patients with positive images were injected earlier after infarction (mean 20.3 ± SD 6.4 hours) compared to those with negative images (27.6 ± 5.8 hours, $p < 0.02$). The other features documented in table 4 (i) age, sex, peak creatine kinase, peripheral white blood cell count, administered dose of ^{111}In and drug therapy did not influence the imaging results.

DISCUSSION

The results confirm that ^{111}In-labelled autologous neutrophils can be used to image the inflammatory response to acute myocardial infarction. Two factors appear to be important in determining whether uptake of radiolabelled neutrophils can be detected within infarcted myocardium.

Firstly, in common with the previous study by Davies et al (Davies, 1981), the time from onset of symptoms to injection with radiolabelled neutrophils appears to be the most important. In their study all patients injected within 24 hours had positive images and similarly in this study all patients injected within 18 hours of the onset

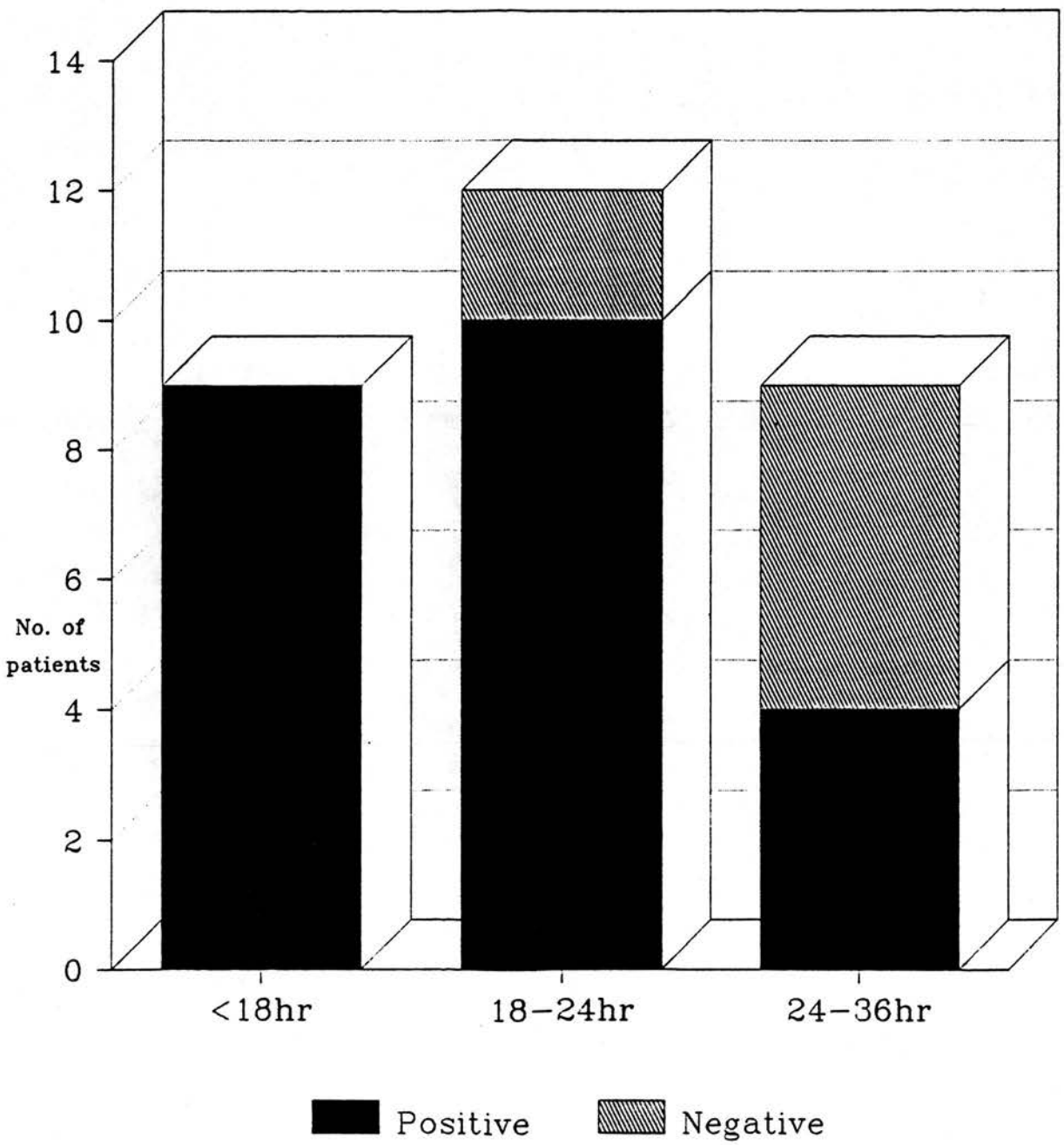


Fig 4h

Bar diagram showing that the percentage of positive images decreases as the interval between the onset of patient symptoms and injection of ^{111}In-labelled neutrophils increases.

of symptoms had positive images. Overall the percentage of positive images was greater in this study (77%) compared to the earlier study (58%) and this in part reflects the earlier mean time of reinjection at 20 hours compared to 43 hours. These results suggest that the stimuli for neutrophil activation and migration are transient, a factor which may be of importance in further studies designed to examine the role of the inflammatory response in the pathophysiology of acute myocardial infarction. Many potential inflammatory mediators may be involved. Components of the complement cascade (C3a and C5a) have been implicated as myocyte mitochondria activate complement (Pinckard, 1975) and complement depletion of animals reduces infarct size as well as the associated inflammatory response (Maroko, 1978). Inhibition of 5-lipoxygenase, the enzyme response for leucotriene B_4 generation, a potent neutrophil chemo-attractant can also reduce the degree of myocardial injury following infarction (Fielder, 1984). Many other inflammatory mediators may be important such as interleukin-1 and platelet activating factor, but as yet no work exists on their role in myocardial infarction. The observations above combined with the evidence that neutrophil depletion reduces infarct size in animal models (Romson, 1983) suggest that activated neutrophils may play a role in extending myocardial injury following myocardial infarction. Similarly, the neutrophil may be important

in reperfusion injury following successful thrombolytic therapy (Braunwald, 1985). If studies are to be designed to further investigate the role of the neutrophil in secondary myocardial injury in man then the imaging results suggest that patients should be recruited within the first few hours of the onset of symptoms. However, they also suggest that it may be possible to influence neutrophil migration for several hours after the onset of symptoms and perhaps as long as 18 hours. Therefore, drugs which influence neutrophil migration to the site of myocardial damage, such as ibuprofen (Romson, 1982) could be administered several hours after infarction while still having a beneficial effect on myocardial salvage. Thrombolytic therapy is the method most commonly used to improve myocardial salvage and although this can be administered 12-24 hours after infarction, the greatest benefit is to patients treated within 4 hours and preferably earlier (GISSI, 1986, 1987; ISIS 2, 1988). Despite the clinical benefits of this therapy there is still discussion about the potential harmful effects of reperfusion, which may be in part mediated through activation of neutrophils (Braunwald, 1985). ^{111}In-labelled neutrophils may therefore provide a method to study the inflammatory response in myocardial infarction in patients treated conventionally or with thrombolytic therapy providing patients were studied early in the course of the infarct and definitely within 18 hours

(Chapter 5).

While the time to injection of the radiolabelled cells in relation to onset of symptoms is important and probably reflects the presence of inflammatory mediators, the other factor which improved the ability to detect myocardial uptake of ^{111}In-labelled neutrophils was the use of single photon emission computed tomography in addition to conventional planar imaging. This improved spatial separation of areas of positive myocardial uptake from uptake in adjacent bone, liver and spleen and with simultaneous blood pool imaging with ^{99m}Tc-human serum albumin allow confirmation of uptake in the region of myocardium. Using this technique 6 patients had positive myocardial uptake of ^{111}In which could not be definitely identified on the corresponding planar images. Myocardial imaging with ^{111}In-neutrophils is not a technique for identifying myocardial infarcts, but single photon emission computed tomography with ^{99m}Tc-pyrophosphate has been used to detect and quantify the extent of myocardial infarction (Jansen, 1985). A similar method is described in the following chapter to assess the extent of uptake of ^{111}In-labelled neutrophils in relation to infarct size.

None of the patient features such as age, sex, peak creatine kinase, peripheral white blood cell count,

administered dose of ^{111}In or concomitant drug therapy appeared to influence the imaging results which supports the suggestion that neutrophil migration is influenced by the extent and duration of release of inflammatory mediators.

This part of the study confirms that it is possible to image the inflammatory response to acute myocardial infarction in man and early injection of labelled cells after the onset of symptoms is important if positive uptake is to be seen. Providing patients are studied early it should be possible to further examine the role of inflammatory response in myocardial infarction and assess the effect of therapeutic interventions such as thrombolytic therapy. This problem is examined further in the following chapter.

CHAPTER 5

THE EFFECT OF TREATMENT WITH AND WITHOUT THROMBOLYTIC THERAPY ON THE INFLAMMATORY RESPONSE AND INFARCT SIZE, ASSESSED BY IMAGING TECHNIQUES, IN PATIENTS WITH ACUTE MYOCARDIAL INFARCTION

Infarct avid myocardial imaging has emerged as a useful non-invasive technique to aid in the detection, localisation and quantification of myocardial infarction (Turi, 1985). In infarct avid imaging a radiopharmaceutical accumulates within a region of damaged myocardium allowing its visualisation as a "hot spot". The most commonly used agent is technetium 99-m stannous pyrophosphate (^{99m}Tc-PYP) first described by Bonte et al in 1974. This radioisotope is normally used for bone scans but is also taken up in damaged myocardium. Uptake within damaged myocardium is dependent on residual blood flow to the damaged area, with ^{99m}Tc-PYP localising to areas of irreversible cell damage associated with elevated calcium levels, in whichever form the calcium is bound (Buja, 1977). Uptake of the radiopharmaceutical within the area of damage begins to increase after 4 hours but scans in general become positive 12-24 hours after infarction (Holman, 1978) and become increasingly abnormal in the initial 24-72 hours, probably as collateral blood flow develops. Successful reperfusion with thrombolytic

therapy leads to early accumulation of ^{99m}Tc-PYP within myocardium and this may relate to the improved blood supply to the area of damage (Wheelan, 1985).

Infarct avid myocardial imaging is used clinically to diagnose myocardial infarction in patients when the electrocardiogram is equivocal as in left bundle branch block, previous infarction, suspected sub-endocardial infarction or following cardioversion or during the immediate post-operative period when enzyme results may be misleading. However, imaging techniques can also be used to assess infarct size which, because of the increasing use of thrombolytic therapy to improve myocardial salvage, is of current experimental and clinical interest. Using planar imaging a crude grading system of myocardial uptake of ^{99m}Tc-PYP (0 to 4) has been devised (Parkey, 1974) which in a large clinical series correlates well with other non-invasive estimates of infarct size (Turi, 1985). Animal models suggest that at least 3 grammes of myocardial necrosis is required to be consistently detected using ^{99m}Tc-PYP and two-dimensional imaging (Stokely, 1976). However, tomographic imaging systems with three dimensional visualisation using single photon emission computed tomography can detect smaller areas of myocardial necrosis (Lewis, 1984). Using this technique estimates of infarct size in man correlate well with infarct size assessed by creatine kinase-MB isoenzymes

(Jansen, 1985). The potential advantage of imaging techniques over other methods is that they need only be performed once whereas serum enzyme analysis requires early and frequent sampling.

Another indirect estimate of infarct size may also be obtained by measuring the left ventricular ejection fraction by equilibrium radionuclide ventriculography (Been, 1986) as it has been shown that ventricular damage relates to the extent of myocardial damage (Hammermeister, 1979).

The results in Chapter 4 show that it is possible to image the inflammatory response to acute myocardial infarction using ^{111}In-labelled autologous neutrophils. However, it is necessary to inject the patients with the labelled cells within 18 hours of the onset of symptoms to ensure positive images. Previous studies have suggested that the neutrophil may extend myocardial injury following myocardial infarction (Romson, 1982; 1983) and may also have a significant role in reperfusion injury (Braunwald, 1985). The role of the neutrophil in myocardial injury in man has not been studied. The object of this part of the study was to compare the degree of inflammatory response with the extent of myocardial injury following myocardial infarction in patients admitted to the coronary care unit using imaging techniques. Radiolabelled

autologous neutrophils were used to assess the inflammatory response, and infarct size was assessed with ^{99m}Tc-PYP. To improve detection and localisation of the radioisotopes and allow quantification, the extent of the inflammatory response and infarct size were measured using single photon emission computed tomography (SPECT). Further indirect estimates of infarct size were obtained from serial measurement of plasma creatine kinase and by assessment of the left ventricular ejection fraction prior to discharge.

Finally, a comparison was also made between patients who were treated conventionally following myocardial infarction and patients treated with thrombolysis, in particular to study the effects of thrombolysis on the degree of inflammatory response in relation to the infarct size.

PATIENTS AND METHODS

Patients

All patients were admitted to the coronary care unit with a first acute anterior myocardial infarction. The diagnosis of myocardial infarction was initially based on the clinical history of ischaemic pain and electrocardiographic evidence of anterior myocardial infarction. The patients were individually assessed by

the coronary care staff and deemed suitable or unsuitable for thrombolytic therapy. Patients were considered ineligible for thrombolytic therapy if admitted greater than 4 hours after the onset of chest pain or who were at risk from systemic anticoagulation, the remaining patients were all given intravenous thrombolysis. All patients gave informed consent for the study. Twenty one patients had a ^{111}In-labelled neutrophil scan followed approximately 24 hours later by a ^{99m}Tc-pyrophosphate scan. Of this group 10 patients were considered unsuitable for thrombolytic therapy and were managed in standard fashion. The other 11 patients were given intravenous thrombolytic therapy within 4 hours of the onset of symptoms and received either streptokinase (Kabivitrum, Middlesex, UK) or anisoylated (human) plasminogen-streptokinase activator complex (APSAC, Beecham Pharmaceuticals, Epsom, UK). The 5 patients given streptokinase were also given 100 mg hydrocortisone and 10 mg chlorpheniramine prior to the 1 hour infusion of 1,200,000 units streptokinase in 100 ml 5% Dextrose. APSAC was given to 6 patients in a dose of 30 units administered as a single intravenous injection over 5 minutes.

Patients given thrombolytic therapy were subsequently anticoagulated with intravenous heparin when the thrombin time became less than 50 seconds and otherwise received

standard care. In addition a further 5 patients who did not receive thrombolytic therapy had a ^{99m}Tc-PYP scan at 48-72 hours and were used for the comparison of infarct size assessed by creatine kinase. Patient details are given in table 5(i) and 5(ii). Twenty patients had a radionuclide ventriculogram to calculate left ventricular ejection fraction prior to discharge.

Cell labelling

On admission to the coronary care unit the patients had a 19 gauge venflon inserted into a forearm vein for venous access and within 16 hours of admission all patients had 60 ml of blood withdrawn into a syringe containing 300 units of preservative free heparin (Leo Laboratories, Buckinghamshire, UK). 50 ml of blood was used to separate and subsequently label neutrophils with ^{111}In-oxine as described in chapter 3. All patients were injected with 5 ml of ^{111}In-labelled autologous neutrophils within 18 hours of the onset of symptoms and imaged the following day. 10 ml of blood was used to measure full blood count and total creatine kinase. Total creatine kinase was measured using a Beckman CK-NAC reagent kit (Beckman Instruments, Carlsbad, USA) adapted for use on a COBA's FARA centrifugal analyser (Roche Diagnostics, Welwyn Garden City, England).

^{111}In-imaging

The patients were imaged approximately 24 hours after the injection of ^{111}In-labelled autologous neutrophils, 10 patients having been treated conventionally and 11 treated with thrombolytic therapy. Imaging was performed using SPECT with the gamma camera (GEC-400AT, Maxicamera) linked to a computer (Microdelta, Siemens). A sequence of 64 two dimensional images was acquired for ^{111}In(energy window 170-278 keV) as the head of the gamma camera rotated through 360^{o}, starting from the right anterior oblique position. Total imaging time was 32 minutes after which the computer summated and reconstructed the 64 projections to provide images in the transverse, saggital or coronal plane.

^{99m}Tc-pyrophosphate imaging

Pyrophosphate imaging was performed in the same patients 24 hours after ^{111}In imaging for the 21 patients who had both ^{111}In and ^{99m}Tc-PYP, and between 48-72 hours post infarction for the remaining 5 patients. All patients had 500 MBq of ^{99m}Tc-PYP injected intravenously 2 hours prior to imaging with SPECT. A sequence of 64 images was acquired for ^{99m}Tc(energy window 129-156 keV) as the head of the gamma camera rotated through 360^{o}. Total imaging time was 12 minutes and summation and reconstruction performed as for indium.

Image interpretation and quantification

Both ^{111}In and ^{99m}Tc images were graded positive when uptake was visible in all three views (ie transverse, saggital and coronal) in the region of the myocardium (fig 5a).

Quantification of the volume of uptake of ^{111}In and ^{99m}Tc was performed on a sequence of transverse slices showing positive uptake for each isotope. Separate regions of interest were drawn for the ^{111}In and ^{99m}Tc images (fig 5b). All transverse slices with positive uptake were then analysed separately using a semi-automatic programme to count the number of voxels (volume cell elements) with uptake of ^{111}In or ^{99m}Tc greater than 65% of the peak myocardial uptake (Jansen, 1985). An individual value was then produced for the volume of myocardium with neutrophil uptake and for the volume of infarcted myocardium for a given patient. The ratio or ^{111}In voxels/^{99m}Tc voxels was used to assess the extent of the inflammatory response in relation to infarct size.

Radionuclide ventriculography

Left ventricular ejection fraction (LVEF) was assessed by gated equilibrium radionuclide ventriculography after the injection of 750 MBq of ^{99m}Tc-labelled human serum albumin in 20 patients prior to discharge. After background correction LVEF was calculated automatically, using a

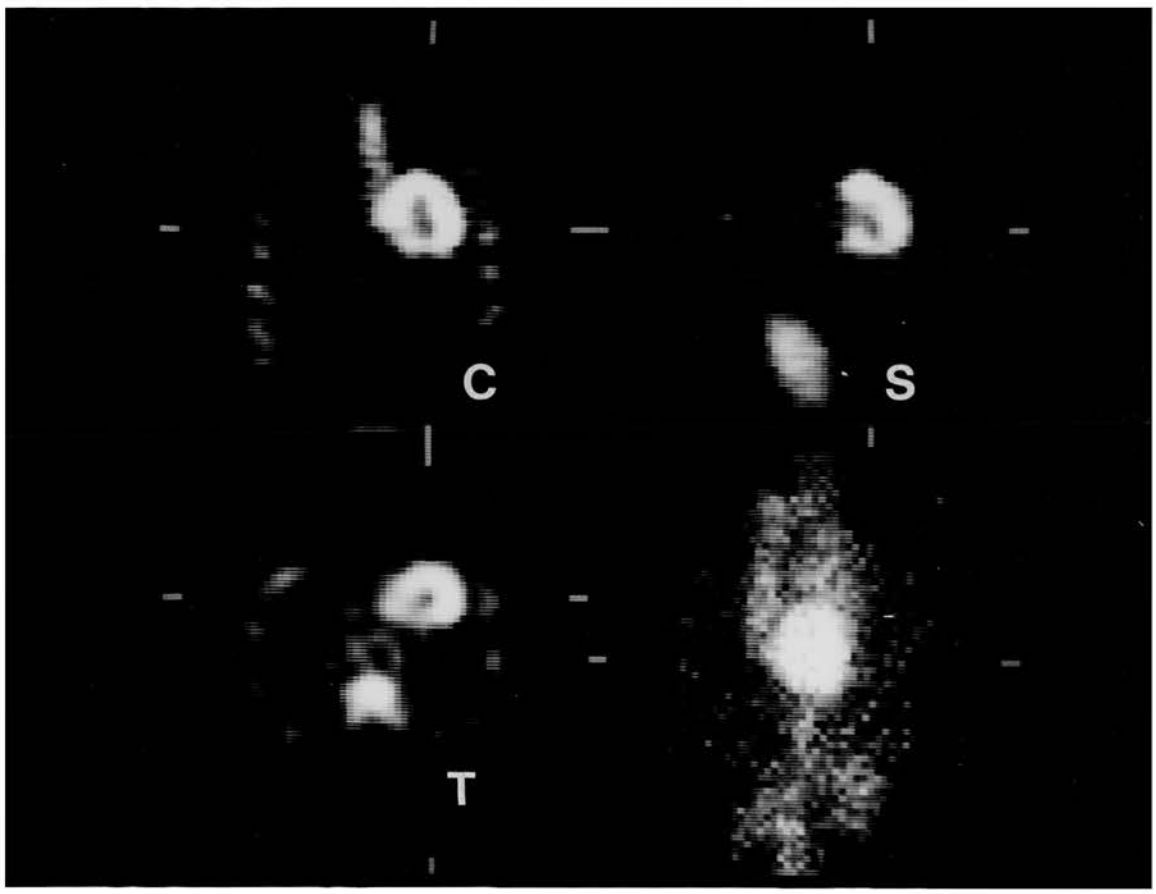


Fig 5a Computer reconstructed images of uptake in the transverse (T) sagittal (S) and coronal (C) planes in a patient with anterior myocardial infarction using ^{99m}Tc-pyrophosphate.

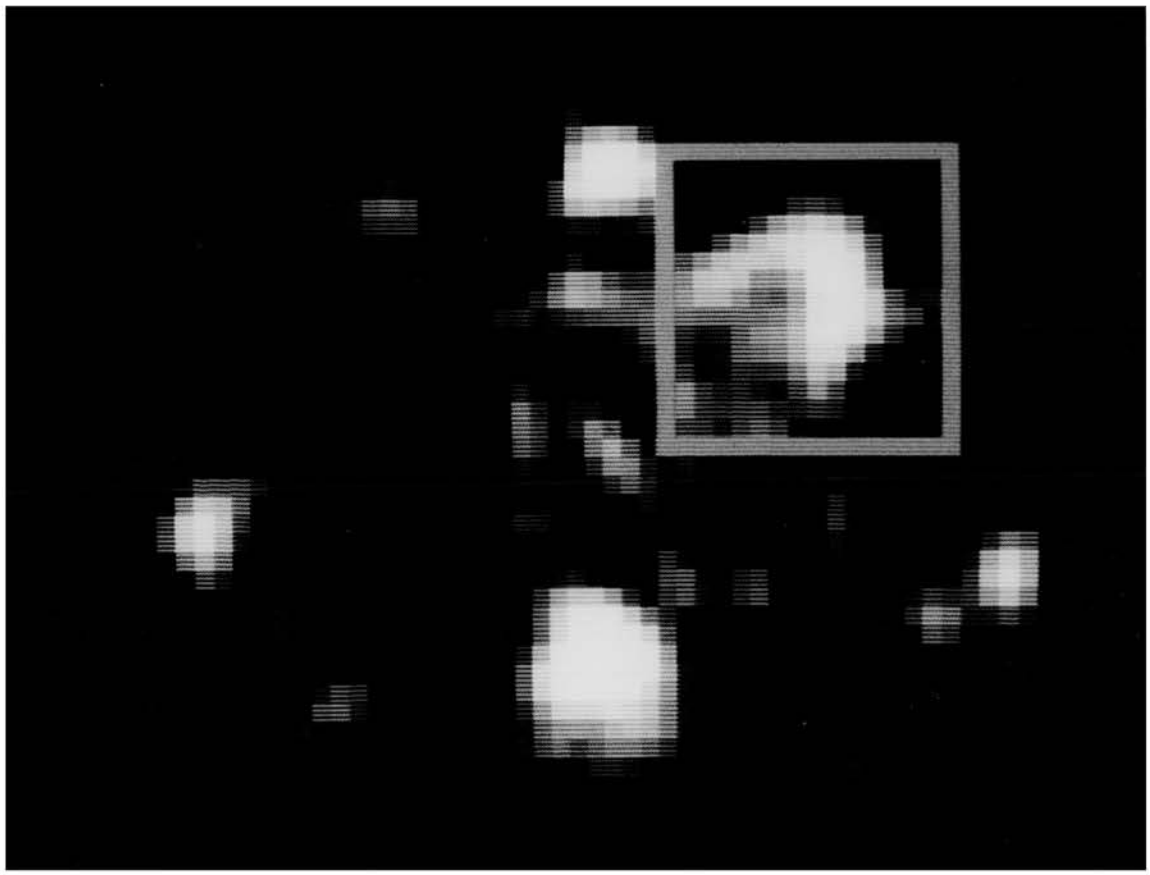


Fig 5b Computer reconstructed image in the transverse plane showing positive uptake of ^{99m}Tc-pyrophosphate within the left ventricle and the region of interest used to calculate the volume of infarct.

region of interest drawn around the left ventricle in end-diastole. Thus

$$\text{LVEF} = \frac{\text{end-diastolic counts - end-systolic counts}}{\text{end-diastolic counts}}$$

STATISTICAL ANALYSIS

The data were not normally distributed and are expressed as median and range. All statistics were performed by non-parametric analysis using the Wilcoxon Rank sum test for two independent samples (Mann-Whitney) or paired samples as appropriate. Values of $p < 0.05$ were considered significant and the data analysed by computer using the statistical package for social sciences (SPSS, Inc, Chicago).

RESULTS

IMAGING

a) **Comparison of inflammatory response (^{111}In) in relation to infarct volume (^{99m}Tc)**

Assessed by ^{111}In-labelled neutrophil imaging and ^{99m}Tc-PYP imaging respectively, the volume of the inflammatory response (85 voxels, range 0-276) was significantly less compared to the volume of infarct (190 voxels, 33-405, $p < 0.0006$) in the 21 patients who had both radioisotope scans

peformed.

b) **Comparison of treatment with and without thrombolytic therapy on inflammatory response and infarct size**

Patient examples showing the effect of conventional therapy and thrombolytic therapy are shown in figure 5d. ^{111}In uptake was slightly larger in patients treated without thrombolysis (114 voxels, range 19-276) compared to patients treated with thrombolytic therapy (81 voxels, 0-160) but this difference was not significant. There was no difference in infarct volume between patients treated without thrombolysis (201 voxels, 77-405) and those given thrombolysis (217 voxels, 111-323). However, the ratio of ^{111}In voxels/^{99m}Tc-PYP was significantly greater in patients treated without thrombolysis (0.79, 0.06 - 2.14) compared to patients treated with thrombolytic therapy (0.41, 0 - 0.96, $p < 0.05$) (fig 5c).

There was no difference in peak creatine kinase between patients treated conventionally (2508 units, 522-6255) compared to those treated with thrombolytic therapy (2500 units, 533-6955). Though LVEF was poorer in patients treated conventionally (36%, 15-51) compared to those given thrombolysis (40%, 27-68), this was not significant. The results and statistics are summarised in table 5(iii).

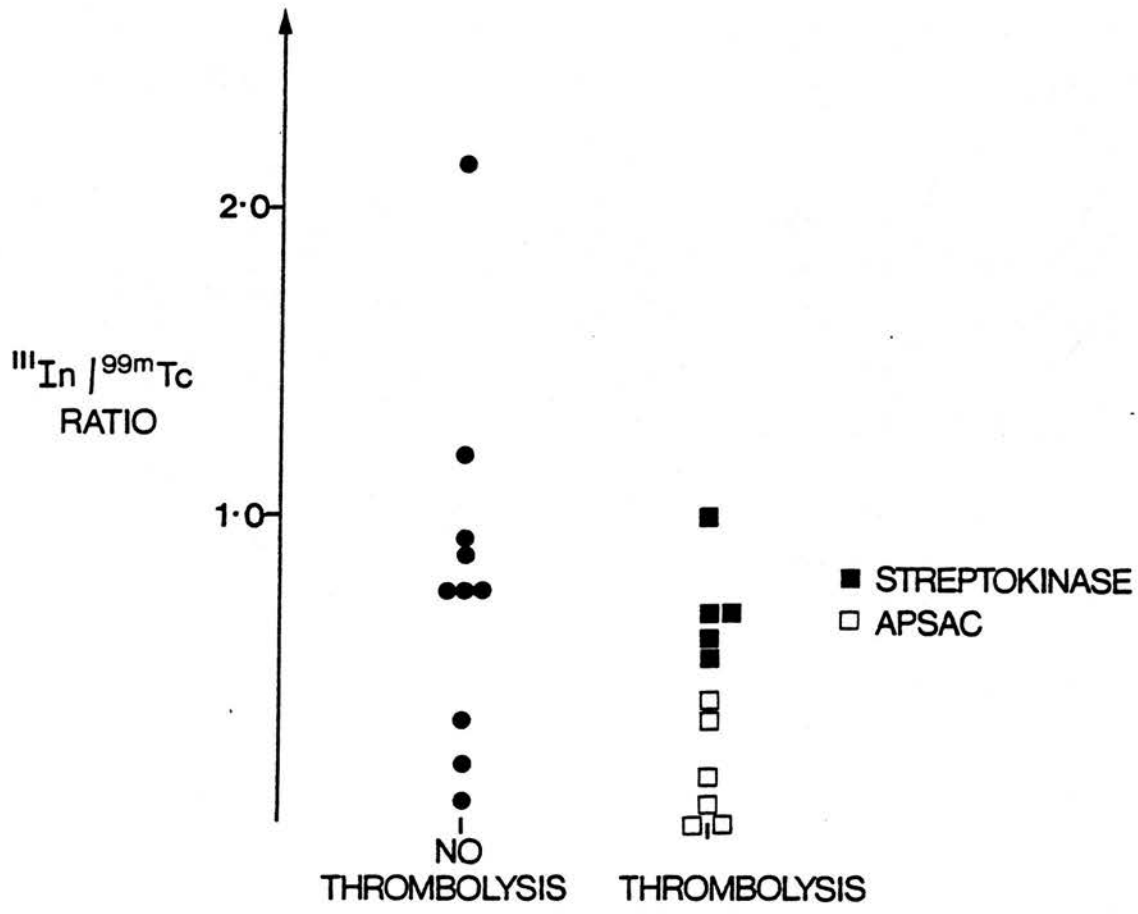


Fig 5c

Diagram showing the extent of uptake of ^{111}In- neutrophils within myocardium expressed as a ratio of the size of myocardial infarction measured by ^{99m}Tc pyrophosphate in patients treated with and without thrombolytic therapy. The ratio is significantly less in patients treated with thrombolysis ($p < 0.05$).

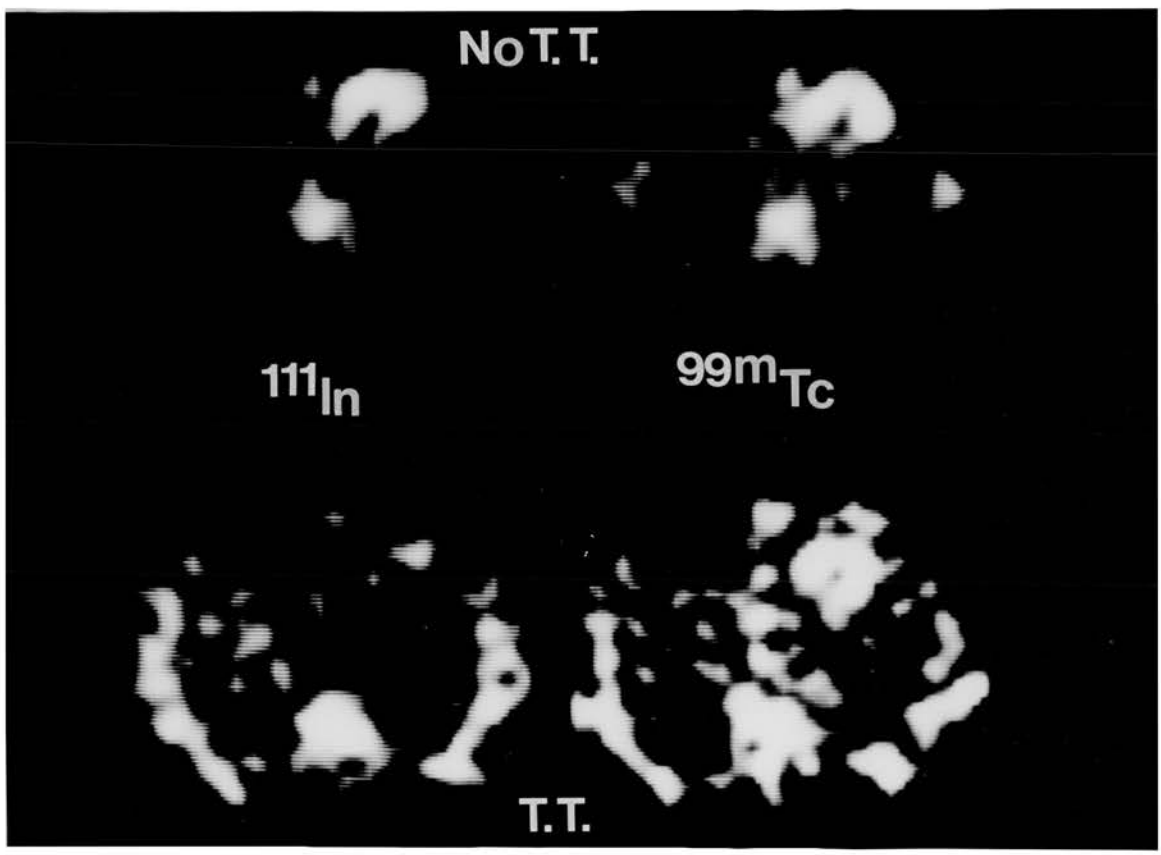


Fig 5d

Single photon emission tomographic images comparing the uptake of ^{111}In-neutrophils with ^{99m}Tc-pyrophosphate. The upper pair of images show easily detected uptake of ^{111}In and ^{99m}Tc-PYP in a patient with anterior myocardial infarction not given thrombolytic therapy. The lower pair of images show markedly reduced uptake of ^{111}In-neutrophils (arrow) compared to ^{99m}Tc-PYP in a patient with anterior myocardial infarction treated with APSAC.

c) Correlation of imaging with ^{111}In and ^{99m}Tc-PYP and estimates of infarct size

There was no correlation between the ^{111}In uptake and the three measures of infarct size; peak creatine kinase ($r = 0.04$), ^{99m}Tc-PYP ($r = -0.03$) and LVEF ($r = 0.34$). There was a correlation between ^{99m}Tc uptake and peak creatine kinase ($r = 0.53$, $p < 0.05$), (fig 5e) and with LVEF ($r = -0.75$, $p < 0.001$) (fig 5f). If the two patients with extremely high values for peak creatine kinase (> 6,000 units), who differed from the remaining patients, are excluded, a sub-group analysis shows an improved correlation between ^{99m}Tc-PYP and peak creatine kinase ($r = 0.78$, $p < 0.001$). The correlation between peak creatine kinase and LVEF is shown in figure 5g.

DISCUSSION

The results confirm the previous observation that inflammatory response to acute myocardial infarction can be reliably imaged using ^{111}In-labelled autologous neutrophils. In only 2 patients could ^{111}In uptake not be identified and both had received thrombolytic therapy. Though these could represent clinical failures, it is likely this is an effect of thrombolysis as the uptake of neutrophils within myocardium appeared less in this group of patients.

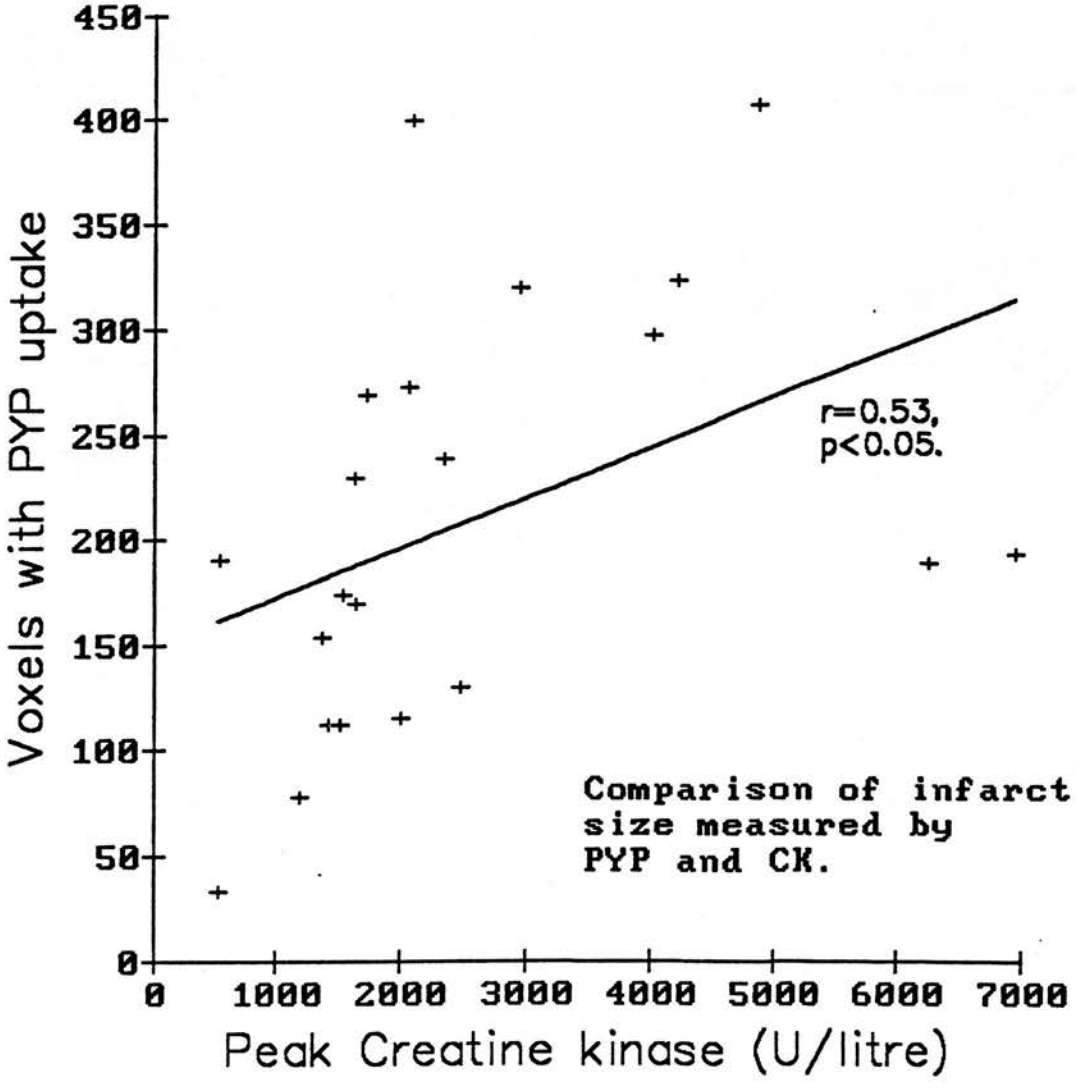


Fig 5e

Figure showing the correlation between peak creatine kinase and volume of infarct measured by uptake of ^{99m}Tc-PYP. Note the two patients with high creatine kinase levels in which ^{99m}Tc-PYP appears to underestimate infarct size.

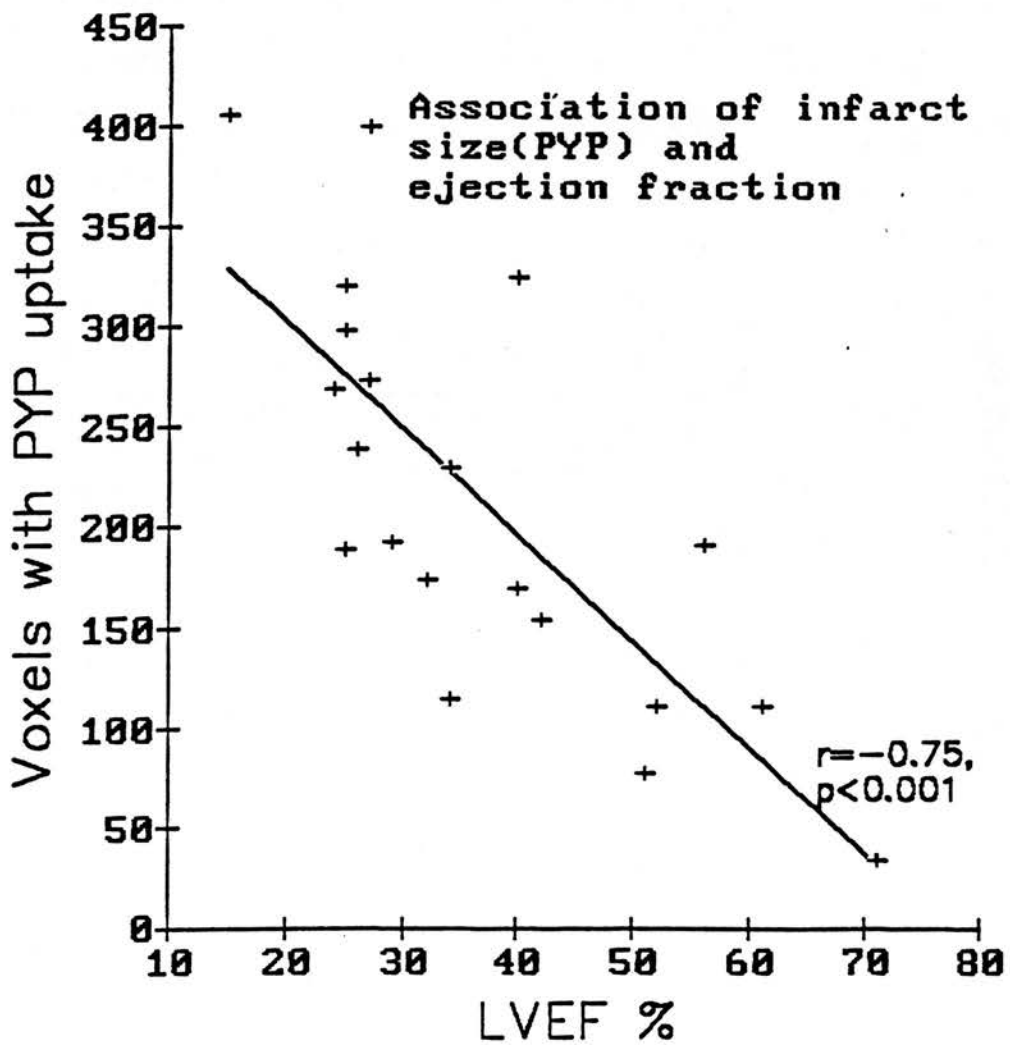


Fig 5f

Figure showing the correlation between infarct size assessed by ^{99m}Tc-PYP and residual left ventricular ejection fraction, calculated by radionuclide ventriculography in patients with anterior myocardial infarction.

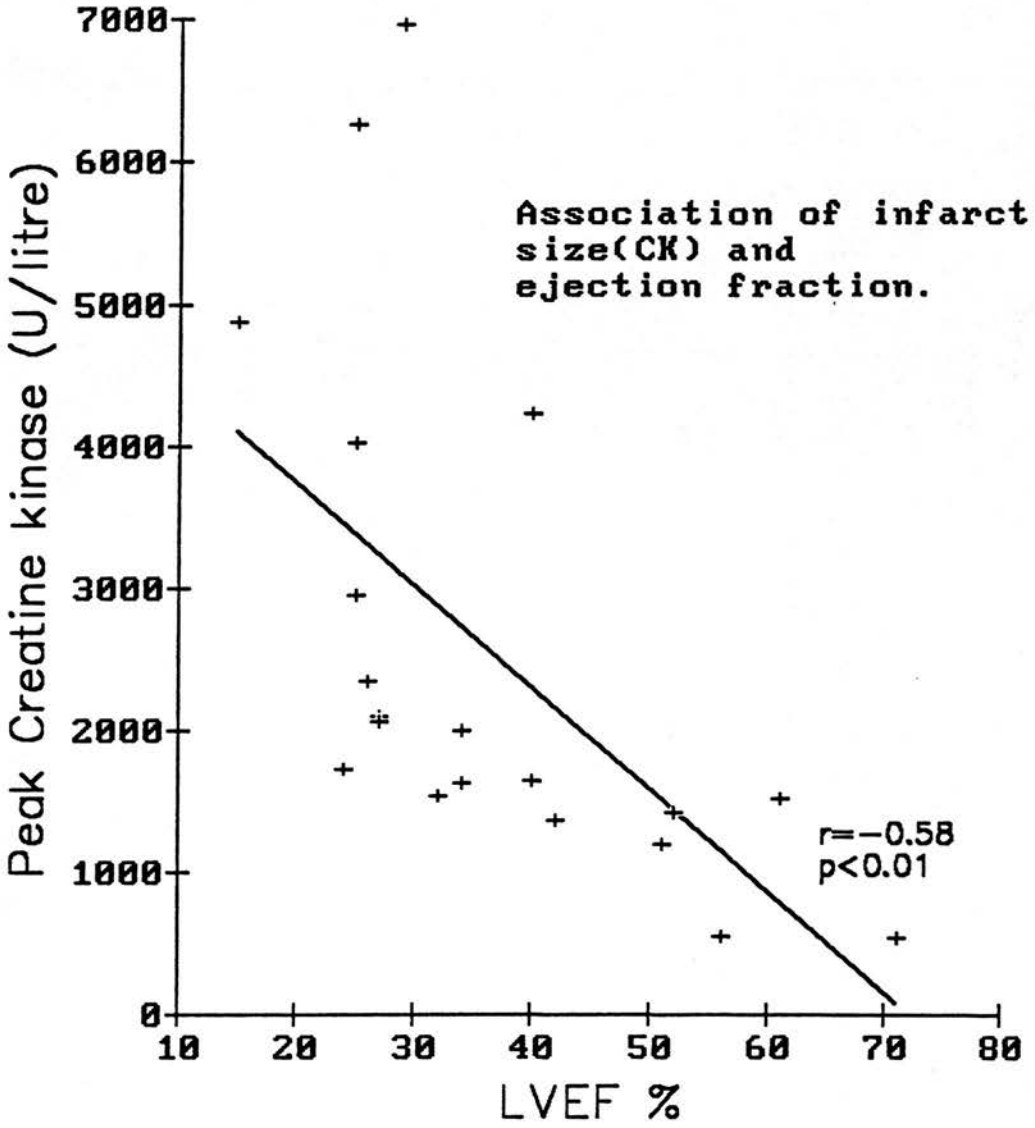


Fig 5g

Figure showing the inverse correlation between peak creatine kinase and residual left ventricular ejection fraction, calculated by radionuclide ventriculography in patients with anterior myocardial infarction.

For all patients, the inflammatory response assessed with ^{111}In-labelled neutrophils, 24-48 hours after infarction, was in general less extensive than the degree of myocardial damage assessed by ^{99m}Tc-PYP approximately 24 hours later. Interestingly, there was no correlation between the extent of ^{111}In uptake and infarct size assessed with ^{99m}Tc-PYP creatine kinase or left ventricular ejection fraction in this group of patients. This confirms that imaging the inflammatory response to myocardial infarction with radiolabelled neutrophils should not be used to detect myocardial infarction. As the neutrophil infiltrate does not correlate directly with the degree of myocardial injury, this suggests other factors influence the extent of the inflammatory response. These may include the rapidity with which a collateral blood supply develops or the rate of growth of new vessels which can start as early as three to four days (Mallory, 1939). However, the results suggest the inflammatory response to myocardial infarction, assessed by ^{111}In uptake, is less in patients treated with thrombolytic therapy, particularly in comparison to the infarct size (fig 5c). Therefore, an alternative explanation which could influence the acute inflammatory response may be the rapidity with which spontaneous reperfusion of the coronary vessel supplying the infarcted area occurs. This hypothesis is compatible with the results of a previous study which has shown that duration of

myocardial ischaemia as well as neutrophil depletion influences infarct size, (Jolly, 1986). In those patients treated with thrombolytic agents it is possible that the drugs themselves have a direct effect on neutrophil activation or release of inflammatory mediators but this has not yet been studied. It is interesting to note that patients given hydrocortisone prior to streptokinase do not appear to have a reduced inflammatory response compared to patients treated with APSAC (fig 5c). This suggests (although the numbers of patients studied were small), the results in the thrombolytic group are not merely influenced by the potential suppressive effect of hydrocortisone on the inflammatory response and neutrophil function (Hart, 1983). Similarly, lignocaine which can also influence neutrophil function, (Goldstein, 1977) did not affect the results.

Measuring infarct size using ^{99m}Tc-PYP is an accepted technique (Turi, 1985), particularly when volume measurements are made with single photon emission computed tomography (Jansen, 1985). In this study, ^{99m}Tc-PYP imaging was intended as an estimate of infarct size to allow a comparison with the extent of the inflammatory response measured similarly with ^{111}In-neutrophils, and was not initially designed to compare infarct size determined by ^{99m}Tc-PYP and SPECT with other estimates of infarct size. However, plasma creatine kinase and

residual left ventricular function were both measured in this study and can be used to indirectly estimate infarct size. As the blood samples for creatine kinase were primarily obtained for diagnostic reasons, the sampling was not frequent enough to allow estimation of total creatine kinase (Sobel, 1972). Neither was quantification of the cardiospecific MB fraction of creatine kinase undertaken (Rodgers, 1977). All enzyme methods of estimating infarct size are subject to criticism as it has been shown that there is considerable variability in the time course of enzyme evolution between individual patients (Yusuf, 1981). In addition, considerable doubts have been expressed regarding the validity both of the use of peak creatine kinase (Sobel, 1972) and estimation of total creatine kinase release (Roe, 1977), as an accurate reflection of infarct size, since both methods involve a gross oversimplification of a complex biological system. The problem is compounded in this study because following successful thrombolytic therapy cardiac associated enzymes appear in the circulation earlier (Shell, 1983), thus increasing the variability of the time course of evolution of these enzymes. Whether this early increased rate of enzyme release following thrombolysis reflects a "washout of enzymes" from reperfused myocardium (Schroder, 1983) or reperfusion damage (Nayler, 1986) is a subject of debate. Recent evidence would, however, suggest that the intensity

of the enzyme release process even following myocardial reperfusion is dependent on infarct size (Van der Learse, 1988). Despite these problems most authors would agree that measurement of cardiac associated enzymes are a useful if indirect estimate of infarct size.

The results from this study show a correlation between infarct size assessed by ^{99m}Tc-PYP using single photon emission computed tomography and peak creatine kinase, though pyrophosphate may be less accurate in patients with very high creatine kinase results. This correlation may well have been better had more frequent samples been taken for creatine kinase to allow more accurate assessment of infarct size. Similarly, there was a good inverse correlation between ^{99m}Tc-PYP estimated infarct size and residual left ventricular fraction in this group of patients with first anterior myocardial infarction. Also in agreement with previous studies, peak creatine kinase inversely correlated with residual left ventricular fraction (Hori, 1979; Dewhurst, 1981). Thus, the combination of ^{99m}Tc-PYP and SPECT does appear to provide a further indirect estimate of infarct size.

In summary, the results show that, following acute myocardial infarction, the extent of the acute inflammatory infiltrate and myocardial infarction can be imaged using ^{111}In-labelled neutrophils and ^{99m}Tc-

pyrophosphate respectively. In general the inflammatory infiltrate appears less extensive than the degree of myocardial damage, particularly in patients who have been treated with intravenous thrombolytic agents, which suggests that successful reperfusion is not associated with an enhanced or prolonged acute inflammatory response. The results are consistent with the neutrophil contributing to secondary myocardial injury, but do not suggest that the neutrophil contributes excessively to reperfusion injury.

CHAPTER 6

PLASMA NEUTROPHIL ELASTASE AS A MARKER OF NEUTROPHIL ACTIVATION FOLLOWING ACUTE MYOCARDIAL INFARCTION: WITH A COMPARISON OF NO-THROMBOLYTIC VERSUS THROMBOLYTIC THERAPY

The elastases constitute a group of proteolytic enzymes produced by a variety of cell types including leucocytes, monocytes, macrophages, platelets, smooth muscle cells, skin fibroblasts and the pancreas. These enzymes differ in their chemical structure, functional and immunological properties, such that it is possible to identify each independently (Janoff, 1985). Human neutrophil elastase is synthesised primarily in the promyelocytes and stored in the cytoplasmic azurophilic (primary) granules of maturing neutrophils. It is a serine protease, with a serine residue in position 195 of the enzymes primary amino acid sequence, and a molecular weight of 33,000 daltons. It is antigenically and biochemically different from other elastases with the possible exception of monocyte elastase which is also a serine protease, though this may merely represent neutrophil elastase which has been bound and subsequently incorporated intracellularly (McGowan, 1983). As monocytes at most contain 3% of the elastase content of neutrophils, the neutrophil is by far the greatest source of enzyme production and release.

Only major stimuli, such as phagocytosis or cell death will result in release of elastase from the primary granules into the surrounding environment (Weissman, 1980), hence the use of this enzyme as a marker of neutrophil activation.

Though the elastases secreted by other cells, such as macrophages, smooth muscle cells and fibroblasts probably have a role in the normal turnover of connective tissue macromolecules, hydrolysis of matrix molecules by neutrophil elastase probably reflects a pathological process (Janoff, 1983), as this enzyme has a wide range of substrates which not only includes elastin but also other structurally important macromolecules such as collagen, proteoglycans and fibronectin. Many other important plasma proteins, such as immunoglobulin, fibrinogen and complement, can also be hydrolysed by this enzyme (Havemann, 1984).

Although it is evident that such large amounts of this potent enzyme within a host defence cell have an important physiological role (Weismann, 1983), excess or uncontrolled release into the cellular microenvironment has been implicated in the pathogenesis of a number of diseases. Neutrophil elastase is considered to be of importance in the development of certain pulmonary diseases including emphysema, adult respiratory distress

syndrome and cystic fibrosis (Janoff, 1985) but has also been implicated in inflammatory bowel disease, rheumatoid arthritis and glomerulonephritis (Malech, 1987). Regulatory control of this potent proteolytic enzyme is by the circulating inhibitory proteins alpha-1 protease inhibitor and alpha-2 macroglobulin plus locally produced low molecular weight regulators (Janoff, 1985). Alpha-1 protease inhibitor, previously called alpha-1 antitrypsin, is the most important regulator of human neutrophil elastase (Carrel, 1986). It is a glycoprotein with a molecular weight of 52,000 daltons, synthesised in the liver, which binds avidly to neutrophil elastase. Alpha-1 protease has a methionine residue at position 358 in the polypeptide chain which combines with the serine hydroxyl group in the active site of neutrophil elastase and thereafter forms an inactive complex. Despite the presence of these inhibitors it is postulated that neutrophil elastase may escape regulation in several ways. Firstly, local enzyme release may overwhelm available inhibitor; secondly, enzyme released in close proximity to its substrates, may then compete locally with the inhibitors for elastase and thirdly, the most important escape mechanism may be the oxidation of the methionine residue, at position 358 by oxygen free radicals, which then renders alpha-1 protease incapable of inactivating human neutrophil elastase (Johnson, 1979). As stimulated neutrophils also release oxygen free radicals they can

create a microenvironment which is capable of oxidatively inactivating alpha-1 protease (Carp, 1980), thus providing an explanation for the secretion of elastase by the neutrophil in the presence of its naturally occurring inhibitor. The mechanisms of oxygen free radical generation by neutrophils will be more fully discussed in Chapter 7.

Neutrophil elastase has been implicated in the pathogenesis of a number of disease conditions (Malech, 1987) and can be measured in a variety of body fluids including blood. In vitro assays of functional neutrophil elastase can be performed after separating neutrophils or neutrophil granules and using synthetic substrates (Bieth, 1974). However, this is not possible in blood or other body fluids as neutrophil elastase will be bound and inactivated by alpha-1 protease or alpha-2 macroglobulin. The techniques currently used to measure elastase in blood are enzyme linked immunosorbent assay (Senior, 1982) or a standard radioimmunoassay method (Plow, 1982; Stockley, 1982), both employing antibody raised against human neutrophil elastase. There has been much work on human neutrophil elastase in inflammatory conditions and pulmonary disease, but although it has been implicated in myocardial injury (Weiss, 1977), this area has been little studied. In vitro studies have shown that neutrophil elastase can directly cause endothelial

damage (Smedley, 1986) and it has been postulated that elastase may contribute to the pathogenesis of atherosclerosis (Robert, 1984). The experimental studies which have implicated the neutrophil in secondary myocardial injury following myocardial infarction and myocardial reperfusion have already been discussed (Romson, 1983; Jolly, 1986). One potential mechanism of myocardial injury is through the uncontrolled release of proteolytic enzymes including neutrophil elastase which can be measuremed in blood and is used as a marker of neutrophil activation (Plow, 1982; Abboud, 1986).

The aim of this study was to measure plasma neutrophil elastase, using a sensitive radioimmunoassay technique, in patients with documented ischaemic heart disease and patients with acute myocardial infarction to assess the extent of neutrophil activation compared to normal control subjects. In addition, the pattern of appearance of the enzyme in plasma following myocardial infarction was assessed and a comparison made between patients treated with and without thrombolytic therapy. The results were also compared with plasma creatine kinase and the degree of peripheral neutrophil leucocytosis.

PATIENTS AND METHODS

Patients (Acute myocardial infarction)

Thirty two patients who had sustained their first acute anterior myocardial infarction were studied, 21 of whom had ^{111}In-labelled neutrophil and ^{99m}Tc-PYP imaging. The diagnosis was made as before on the history of prolonged ischaemic chest pain and characteristic electrocardiographic changes with a subsequent rise in cardiac associated enzymes including total creatine kinase. On admission 17 patients were considered eligible for treatment with 1,200,000 units streptokinase (pre-treated with hydrocortisone and chlorpheniramine) or 30 units aniisoylated plasminogen streptokinase complex (APSAC). The other 15 patients were considered ineligible for thrombolytic therapy because of late admission (greater than 4 hours after the onset of symptoms) or who were considered at risk from systemic anticoagulation. These patients were treated in conventional fashion, receiving standard drug therapy as indicated. The details of the two patient groups are summarised in table 6(i).

Protocol (acute myocardial infarction)

On admission to the coronary care unit a 19 gauge venflon was inserted into a forearm vein, flushed with 2 ml 0.9% saline and used for all subsequent blood sampling. Blood

sampling was performed 6-8 hours after the onset of symptoms and every 8 hours thereafter for the first 48 hours. The initial sample included a full blood count as well as blood for total creatine kinase, and human neutrophil elastase. Samples were also taken to measure lipid oxidation products as a marker of free radical activity and this will be discussed in chapter 7. All samples were centrifuged immediately at 2000 rpm for 10 minutes at 4°C, the plasma separated and frozen at -20°C until the assays were performed.

Patients also had a resting left ventricular ejection fraction measured prior to discharge.

Patients and Protocol (controls and ischaemic heart disease)

Blood was taken for full blood count and human neutrophil elastase from 30 patients with a documented history of ischaemic heart disease, based on coronary angiography (12) or distant myocardial infarction and from 35 healthy volunteers from laboratory and hospital staff. Details of these groups are given in table 6(i). Again samples for human neutrophil elastase were centrifuged immediately and the plasma frozen at -20°C until the assay was performed.

Methods

Full blood count was measured automatically on a Sysmex E 5000 (TOA Electronics Limited, Kobe, Japan) and a differential count obtained. Creatine kinase was measured as described previously in chapter 5.

Human neutrophil elastase was measured in the Medical Research Council/Scottish National Blood Transfusion Service laboratory by Dr J Dawes. The assay used was a standard radioimmunoassay using specific rabbit polyclonal antiserum raised against human neutrophil elastase (Plow, 1982). The antigen having been purified from human neutrophils following leukopheresis. The antibody is specific for neutrophil elastase and does not cross react with pancreatic or platelet elastases. The assay will measure neutrophil elastase as the free enzyme or complexed with its natural inhibitors alpha-1 antitrypsin and alpha-2 macroglobulin. For the assay, 50 ul of standard/sample was added to 50 ul ^{125}I-elastase plus 50 ul anti-elastase antibody (dilution 1:3000) and made up to 200 ul with buffer comprising 0.05 M phosphate (pH 7.4), 0.6 M NaCl and 2 mM EDTA, 130 mg/ml herparin, 20 units/ml aprotonin and 2% heat inactivated serum. The samples were then incubated overnight at room temperature and separated with donkey anti-rabbit immunoglobulin immobilised on Sepharose. After shaking for 45 minutes at room temperature, the bound complex was separated from

free by sedimentation under gravity through 10% sucrose solution, then aspirated and counted on a gamma counter (NE 1600). The standard curve was constructed from 8 doubling dilutions of fresh frozen plasma and the sensitivity of the assay was 0.5 ng/ml. Intraassay coefficient of variation was less than 5%.

STATISTICS

The data were found not to be normally distributed as tested by the Kolmogorov-Smirnov test. Results are therefore expressed as median and range. Non-parametric analysis was performed using the Wilcoxon rank sum test for two independent variables (Mann-Whitney) values of $p < 0.05$ were assumed to be significant and all data were analysed by computer using the statistical package for social sciences (SPSS Inc, Chicago, USA).

RESULTS

Comparison of control subjects with patients with ischaemic heart disease and acute myocardial infarction

White cell count The white blood cell count was significantly lower in the normal volunteers (median 5.8 x 10^9/litre, range 3.4-9.3) than in patients with ischaemic heart disease without recent infarction (6.7 x 10^9/litre,

5.2-12.6, $p < 0.004$) and both were lower than the initial white cell count in patients with acute myocardial infarction (16.4 x 10^9/litre, 7.9-33.7, $p < 0.0001$). The neutrophil count was also lower in normal volunteers (3.6 x 10^9/litre, 1.8-6.2) compared to patients with ischaemic heart disease (4.7 x 10^9/litre, 3.4-10.4, $p < 0.004$). Both were lower than the initial neutrophil count in patients with acute myocardial infarction (13.0 x 10^9/litre, 6.7-31.7, $p < 0.0001$) (fig 6a).

Plasma neutrophil elastase Compared to the normal volunteers (18.6 ng/ml, 9.2-50.1) plasma neutrophil elastase was significantly higher in the patients with ischaemic heart disease (25.8 ng/ml, 12.2-49.5, $p < 0.02$). The pattern of evolution of plasma neutrophil elastase was different in patients treated conventionally or with thrombolysis following acute myocardial infarction (see below), therefore the results were analysed separately.

For both groups of patients with myocardial infarction the plasma neutrophil elastase was significantly greater between 8 and 48 hours compared to normal control subjects.

For patients treated conventionally, i.e no thrombolytic therapy, the plasma neutrophil elastase was significantly greater between 16 and 48 hours, but not at 8 hours,

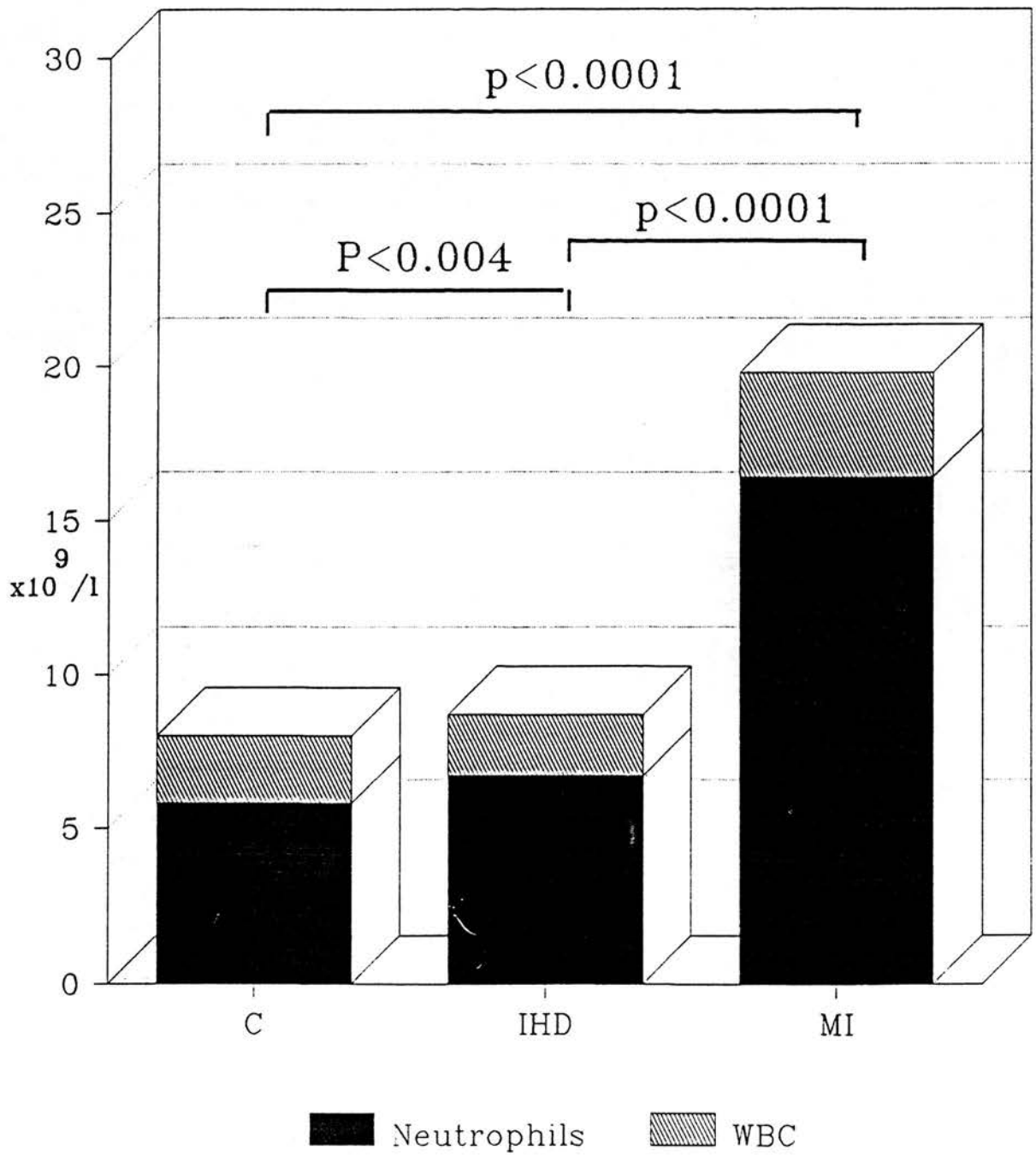


Fig 6a Total white cell and neutrophil counts in peripheral blood in control subjects (C), patients with ischaemic heart disease (IHD) and initial count in patients with acute myocardial infarction (MI).

compared to patients with ischaemic heart disease.

Patients treated with thrombolysis had significantly higher levels of plasma neutrophil elastase between 8 and 40 hours, but not at 48 hours. The statistical results are summarised in table 6 (ii and iii) and the individual data shown in figures 6b and 6c.

Comparison of patients with myocardial infarction treated with and without thrombolysis

Plasma neutrophil elastase The pattern of plasma neutrophil elastase over 48 hours differed in the two groups of patients with acute myocardial infarction and this is shown diagrammatically in fig 6d. Patients given intravenous thrombolysis had initial values (48.2 ng/ml, 25-250) which were significantly greater than patients treated conventionally (32.6 ng/ml, 15.6-101; $p < 0.025$). Those treated conventionally had, as a group, a later peak at 40 hours (49.8 ng/ml, 21.4-196) which was significantly greater than those treated with thrombolysis (34.2 ng/ml, 15.8-83; $p < 0.037$).

Infarct size There was no significant difference in the peak creatine kinase between patients treated conventionally (1635 u/litre, 522-6255) compared to those treated with thrombolysis (2059 u/litre, 533-6955). As a group the peak creatine kinase occurred earlier in those

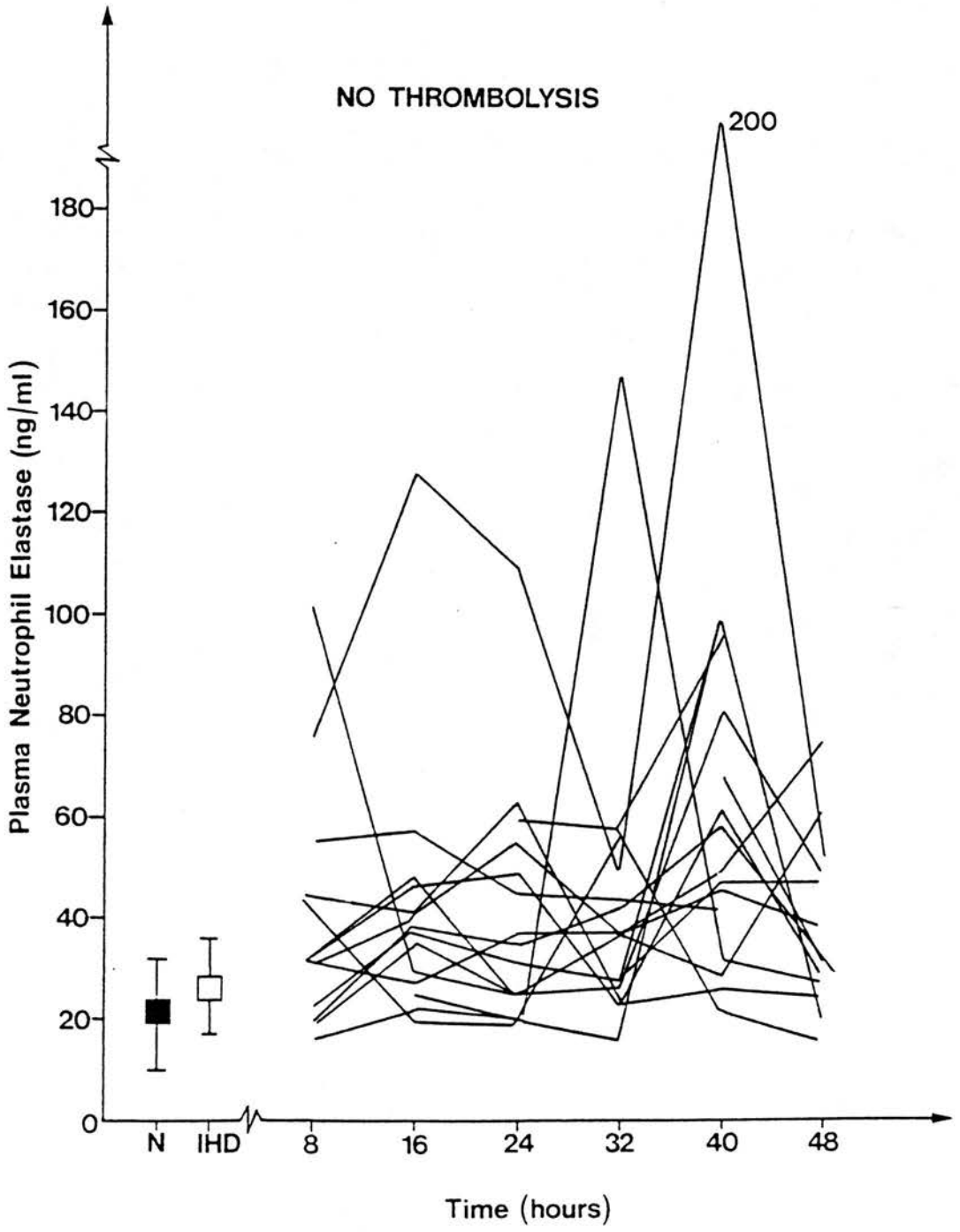


Fig 6b

Individual results for plasma neutrophil elastase between 8 and 48 hours in the patients with acute myocardial infarction who did not receive thrombolytic therapy. Mean values for normal controls (N) and patients with ischaemic heart disease (IHD) are also shown.

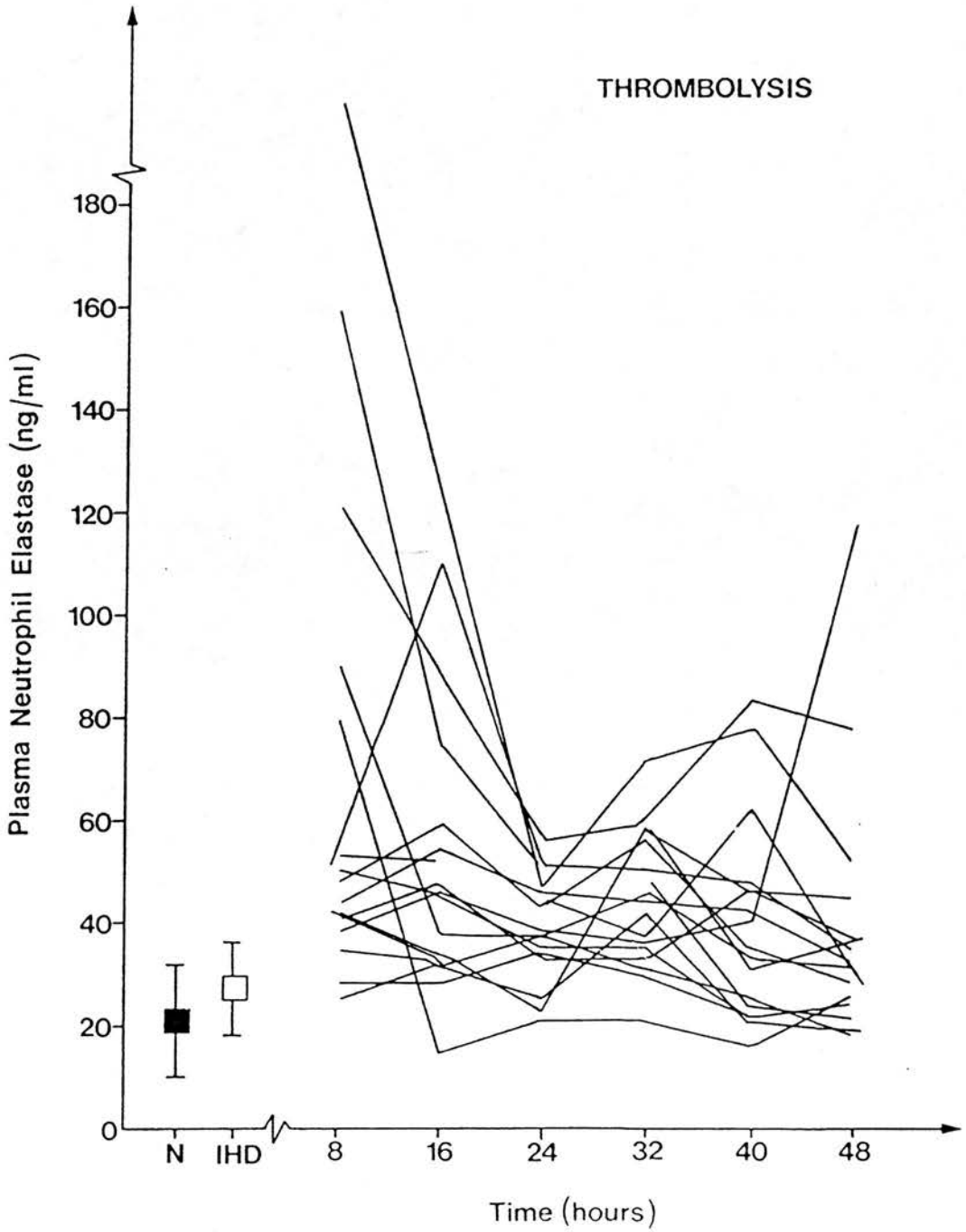


Fig 6c

Individual results for plasma neutrophil elastase between 8 and 48 hours in patients with acute myocardial infarction who were treated with intravenous streptokinase or APSAC. Mean values for normal controls (N) and patients with ischaemic heart disease (IHD) are also shown.

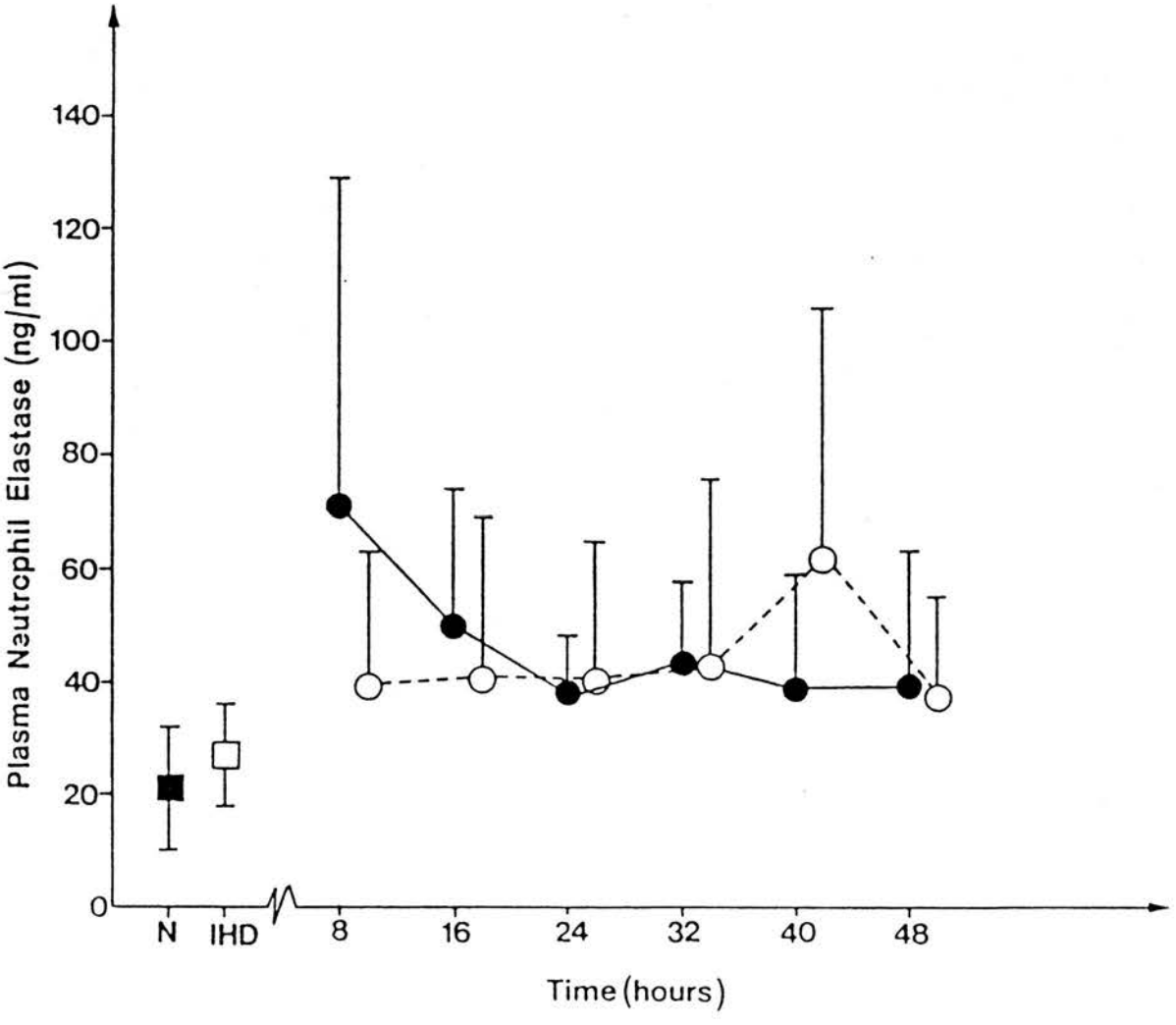


Fig 6d

Mean values (+ 1SD) for plasma neutrophil elastase (PNE) in patients treated with (•) and without (o) thrombolytic therapy following acute myocardial infarction. Levels of PNE are significantly different between the thrombolytic and non-thrombolytic group at 8 hours ($p < 0.025$) and 40 hours ($p < 0.037$).

treated with thrombolysis (16 hours) compared to those patients given thrombolysis (24 hours). Similarly there was no significant difference in the left ventricular ejection fraction measured by radionuclide ventriculography, 10 days post-infarction, though patients treated conventionally tended to have a lower LVEF (32%, 15-71) compared to those treated with thrombolysis (40%, 24-68).

There was no correlation between white blood cell count or neutrophil count with plasma neutrophil elastase in the normal volunteers, patients with ischaemic heart disease or the initial value for patients with acute myocardial infarction.

There were weak correlations between creatine kinase and plasma neutrophil elastase at 8 hours ($r = 0.37$, $p < 0.036$) 16 hours ($r = 0.47$, $p < 0.011$), 24 hours ($r = 0.53$, $p < 0.0028$) and 40 hours ($r = 0.39$, $p < 0.03$).

DISCUSSION

White first recognised the association between the extent of myocardial injury and degree or duration of leucocytosis, which reflects the host inflammatory response to tissue damage (White, 1926). More recent studies have also shown an association between leucocyte

count and ischaemic heart disease. In a study of 464 patients who subsequently developed a first myocardial infarction the leucocyte counts, measured on average 17 months earlier, were significantly greater compared with two control groups, though this in part related to cigarette smoking (Friedman, 1974). A more recent study suggests that the risk of myocardial infarction is approximately four times as great in persons with white blood cell counts high in the normal range (greater than 9 x 10^9/litre) compared to those with lower white blood cell counts within the normal range (less than 6 x 10^9/litre) and only 50-65% of this increased risk could be explained by cigarette smoking (Ernst, 1987). This study, though in a smaller group of patients with ischaemic heart disease similarly shows that both the white blood cell count and neutrophil count are increased compared to normal non-smoking volunteers. In the patients with acute myocardial infarction the early leucocytosis is part of the normal response to stress, with catecholamine release, resulting in neutrophils being mobilised from the marginated pool within the pulmonary circulation (Bierman, 1952). However, stress does not explain the results in the patients with ischaemic heart disease, as only 3 admitted to angina pectoris on the same day as blood was taken and all denied current smoking, although carboxyhaemoglobin was not measured to verify this. An increased white cell count may be an important risk factor

in myocardial infarction as neutrophils contribute significantly to the rheological properties of blood, particularly when activated as they become more adherent to the endothelial wall, producing impaired blood flow within the microcirculation (Kloner, 1974). Adherent neutrophils may also contribute to endothelial damage by the release of elastase (Smedley, 1986).

The results show that plasma neutrophil elastase is mildly elevated in patients with stable ischaemic heart disease. However, within the circulation, neutrophil elastase is inactive, as it is complexed to the circulating inhibitors alpha-1 protease and alpha-2 macroglobulin. These inhibitors are present in much greater quantities in the circulation, in gram quantities compared to nanogrammes of elastase and, therefore, although the increase in plasma neutrophil elastase reflects neutrophil activation does not necessarily imply involvement in the pathogenesis of disease. However, the inhibitory mechanisms may not operate within the immediate microenvironment of the cell (Janoff, 1985) and activated neutrophils are capable of producing in vitro endothelial damage via elastase release (Smedley, 1986). Thus, in patients with stable ischaemic heart disease the elevated levels of neutrophil elastase may reflect neutrophil activation in proximity to endothelial cells with subsequent endothelial damage (Robert, 1984) but equally may be a non-specific marker of

pre-existing atheroma with possible mechanical damage to the circulating neutrophil.

In patients with acute myocardial infarction, plasma neutrophil elastase is significantly elevated for at least the first 40-48 hours following the onset of symptoms. This increase could be interpreted as an acute phase response to stress as occurs with alpha-1 antiprotease and other acute phase proteins (Laurell, 1975). However, this is unlikely to explain the results for two reasons. Firstly, the leucocytosis seen following myocardial infarction, though part of the normal stress response, does not correlate with plasma neutrophil elastase. Secondly, the pattern of evolution of neutrophil elastase differs between patients treated conventionally compared to those given thrombolytic therapy, while both groups are presumably exposed to similar degrees of stress following myocardial infarction. This variation suggests that the reasons for neutrophil activation may be different in the two groups of patients.

In the patients treated without thrombolysis the individual results suggest that although neutrophil elastase is elevated, the values remain relatively constant over the first 24 hours with later peaks at 32 and 40 hours. This coincides with neutrophil infiltration into the area of damaged myocardium

demonstrated by earlier histological studies (Mallory, 1939; Sommers, 1964), which can also be imaged within 48 hours of infarction using ^{111}In-labelled autologous neutrophils (Bell, 1987). The elevated plasma neutrophil elastase may therefore reflect elastase, complexed to alpha-1 antiprotease, which has been released locally within the myocardium, by activated neutrophils, and subsequently re-enters the circulation.

In contrast, those patients given intravenous thrombolytic therapy, had higher values of plasma neutrophil elastase initially which tended to fall thereafter. These results agree with a recent publication (Gutteridge, 1988) and suggests an alternative mechanism may account for the early release of elastase, as few neutrophils will have migrated into the area of myocardial injury by this stage. Following thrombolysis elastase could be released from neutrophils trapped within the coronary thrombus or from neutrophils activated while adherent to the endothelium of the reperfused coronary vessel (Kloner, 1974). The previously discussed imaging results would support this concept in that neutrophil infiltration appears less in patients given thrombolysis (chapter 5) and as a group they show little if any late rise in neutrophil elastase. This suggests that the early increase in elastase following thrombolytic therapy reflects intravascular activation of neutrophils rather than neutrophil

degranulation within the ischaemic myocardium.

Another explanation for the different pattern of neutrophil elastase release in the two groups could have been the additional drugs administered to the patients, particularly lignocaine (Goldstein, 1977) and hydrocortisone (Hart, 1984) which both reduce neutrophil activation and lysosomal enzyme release in vitro. It seems unlikely that lignocaine given for ventricular tachycardia significantly affected the results as only 8 of 32 patients were treated in this manner, 3 treated conventionally and 5 treated with thrombolysis. In contrast half of the patients in the thrombolytic group received streptokinase and hence were also given hydrocortisone. A subgroup analysis of the patients given thrombolysis did not show any difference in elastase release between patients treated with streptokinase or APSAC, and thus does not suggest that hydrocortisone influenced the results. Again the imaging results support this as patients given hydrocortisone prior to streptokinase therapy did not have a greater reduction in the uptake of ^{111}In-labelled autologous neutrophils (chapter 5) when compared with patients treated with APSAC. Previous studies examining the role of steroids in myocardial infarction have used much larger doses of methylprednisolone (Roberts, 1976; Kloner, 1978).

The pattern of neutrophil elastase release was not identical for individual patients in either the non-thrombolytic or thrombolytic groups. Such differences may be the result of spontaneous reperfusion in the non-thrombolytic group and failed thrombolysis in the treated group. It is known that the spontaneous reperfusion rate following myocardial infarction is of the order of 20-30% (De Wood, 1980). Therefore, of the group of 15 patients treated conventionally one would expect 3-5 to show spontaneous reperfusion, perhaps marked by an early neutrophil elastase peak; such an early peak of elastase was, in fact, seen in 2 patients. Similarly, we know that intravenous thrombolytic therapy is successful in achieving coronary reperfusion in approximately 60-70% of cases (Laffel, 1984). If failed reperfusion is marked by a late neutrophil elastase peak, as in patients treated conventionally, we would expect a late peak in approximately 5-7 patients treated with thrombolysis and five patients demonstrate an obvious late peak. Unfortunately, as patients did not undergo early angiography it is not possible to confirm this hypothesis.

The results do, however, confirm that the white blood cell, and particularly the neutrophil count is elevated in patients with ischaemic heart disease. Not surprisingly there is a leucocytosis following acute myocardial infarction, but the white cell count also tended to be

high, although within the normal range, in patients with stable ischaemic heart disease. This does not determine cause or effect, but it is interesting that, as well as following myocardial infarction, in stable ischaemic heart disease there is evidence of neutrophil activation as measured by neutrophil elastase. The increase in plasma neutrophil elastase persisted for the first 48 hours following myocardial infarction. In patients treated conventionally the increased elastase occurs when there is known to be a significant neutrophil infiltrate within the myocardium and may therefore be a mechanism of secondary myocardial injury (Weiss, 1977). A late increase in neutrophil elastase is not apparent in most patients treated with thrombolysis who in general show an earlier increase in plasma levels which may reflect intravascular rather than extravascular elastase release from activated neutrophils. The results therefore suggest that neutrophil elastase may have a role in delayed secondary myocardial injury in patients not given thrombolytic therapy. In conjunction with the imaging results which show successful thrombolytic therapy is not associated with a greater neutrophil infiltrate at the site of myocardial injury, the results suggest that neutrophil does not play a major part in reperfusion injury.

CHAPTER 7

LIPID OXIDATION PRODUCTS AS A POTENTIAL MARKER OF OXYGEN FREE RADICAL ACTIVITY FOLLOWING MYOCARDIAL INFARCTION: WITH A COMPARISON OF NO-THROMBOLYTIC THERAPY VERSUS THROMBOLYTIC THERAPY

A free radical is defined as an atom or molecule capable of independent existence which has one or more unpaired electrons, and as such is an unstable chemically reactive species. Within this broad definition there are many free radicals in chemistry and biology including the oxygen molecule, a hydrogen atom and most of the transition metal ions (Halliwell, 1985). Although oxygen has two electrons it behaves like a bi-radical because it has two unpaired electrons of parallel spin, therefore any incoming pair of electrons attempting to react with oxygen would need antiparallel spins and require one of the electrons to undergo a spin inversion. To avoid this "spin restriction", oxygen prefers to accept electrons one at a time. Although this has major advantages in the aerobic environment, as it slows down the reaction with non-radicals, the disadvantage is that electrons when added singly to oxygen lead to the formation of reactive intermediates. When a single electron is added to the ground state O_2 molecule, the product is superoxide radical O_2^-. Within biological systems further reduction

of oxygen can occur with the addition of two and four electrons in the presence of hydrogen, which results in the formation of further reactive intermediates two of which are free radicals (see below).

$$O_2 + e + H^+ \longrightarrow HO_2^{\cdot} \longrightarrow H^+ + O_2^-$$

$$O_2^- + e + 2H^+ \longrightarrow H_2O_2$$

$$H_2O_2 + e \longrightarrow OH^- + \cdot OH$$

$$\cdot OH + e + H^+ \longrightarrow H_2O$$

Superoxide radical is produced in numerous biological reactions, including the electron transport chain of mitochondria and endoplasmic reticulum, where electrons can "leak" into oxygen (Nohl, 1978), by activated phagocytic cells following the respiratory burst (Babior, 1978) and in the presence of ischaemia following the conversion of xanthine dehydrogenase to xanthine oxidase (McCord, 1985). These mechanisms are outlined in fig 7 (a). Once generated superoxide radical can assist the formation of more damaging radical species by "Fenton Chemistry" as shown in the equation below.

$$2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

$$O_2^- + Fe^{3+} \rightleftharpoons (Fe^{3+}\text{-}O_2^- \leftrightarrow Fe^{2+}\text{-}O_2) \rightleftharpoons (Fe^{2+} + O_2)$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + \cdot OH$$

This results in the generation of hydroxyl radical ($\cdot OH$)

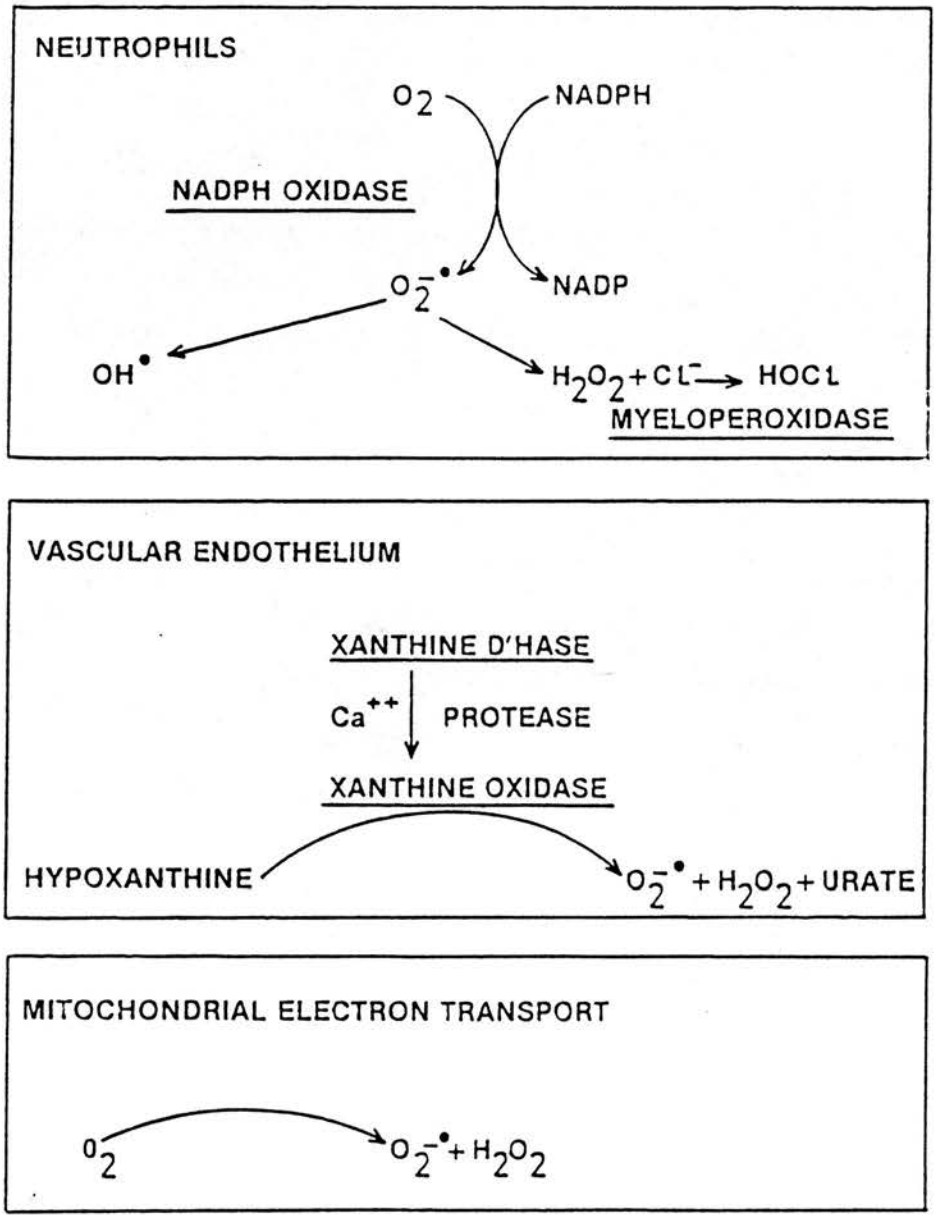


Fig 7a Proposed sources of oxygen derived free radicals and toxic oxygen metabolites in myocardial ischaemia. 1) Neutrophil-membrane associated NADPH oxidase reduces molecular oxygen initially to superoxide anion (O_2^-) with production of hydroxyl radical (OH) and hydrogen peroxide (H_2O_2) which is converted to hypochlorous acid by myeloperoxidase. 2) Vascular endothelium: with ischaemia xanthine dehydrogenase is converted to an oxidase with accumulation of hypoxanthine and production of O_2^-. 3) Mitochondrial electron chain transport - small amounts of oxygen free radicals are normally produced but this increases in the presence of ischaemia.

which is highly reactive and capable of damaging most biological molecules. In addition to the inorganic oxygen radicals, organic oxygen radicals can be produced which are equally important biologically. Probably the most studied organic oxygen radical reactions are the autooxidation products of polyunsaturated fatty acids, more commonly known as lipid peroxidation (Halliwell, 1985). Lipid peroxidation proceeds by a chain reaction and polyunsaturated lipids are particularly sensitive to free radical attack since they contain methylene groups in interrupted double bond systems whose hydrogen atoms are susceptible to abstraction. Such a reaction generates a lipid conjugated diene which in the presence of oxygen can react further to form hydroperoxides (fig 7b). Alternatively the outcome is the formation of malonaldehyde. Lipid hydroperoxides can be decomposed by transition metal ions, such as iron to give alkoxy and peroxy radicals which ultimately yield numerous carbonyl compounds. This pathway may account for the stimulation of lipid peroxidation following tissue damage, as metal complexes may be released from vacuoles, organelles or from other sequestered sites within the cell. It may also explain the frequent observation that lipid peroxidation accompanies many disease processes associated with cellular injury. Lipid peroxides have themselves been implicated in vascular endothelial damage (Blake, 1985) and in myocardial injury (Meerson, 1982), though the

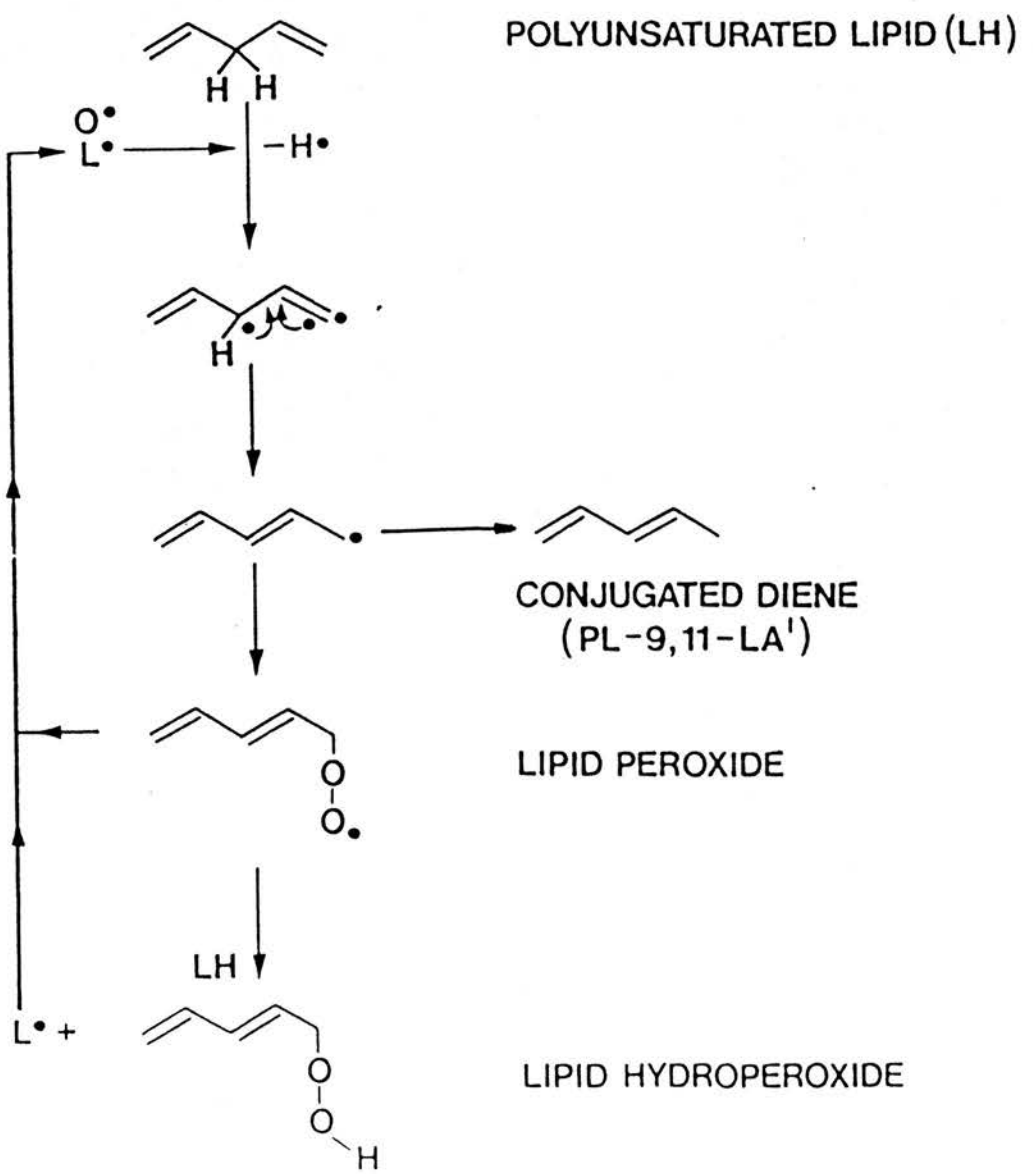


Fig 7b Diagrammatic representation of the pathway of lipid peroxidation for a polyunsaturated fatty acid such as linoleic acid.

interpretation that cell damage can be caused by lipid peroxidation has been questioned (Halliwell, 1984). In addition, they may also regulate prostaglandin biosynthesis (Lands, 1984).

Oxygen radicals are produced by numerous biological reactions and are part of the normal physiological process of cell metabolism in which oxygen plays a crucial role, particularly in a number of highly organised enzyme systems. However, free radicals if unimpeded will result in denaturation of proteins, peroxidation of membrane lipids and insterstitial matrix damage producing altered enzyme activity and membrane permeability (McCord, 1985). Fortunately, there are a number of endogenous antioxidant mechansims found intracellularly and extracellularly which directly or indirectly protect the host from free radical mediated damage including enzymes such as superoxide dismutase and vitamin C,table. 7 (i).

Free radicals are inherently unstable and reactive chemical species and characterstically therefore free radical activity is extremely difficult to measure as they rapidly react with and oxidise adjacent molecules (Dormandy, 1984). Most methods, are therefore indirect and measure free radical oxidation products, which include the oxidation products of proteins measured by the fluorescence/ultraviolet absorbance ratio of

immunoglobulin G (Wickens, 1983) and the oxidative products of lipids. Quantitative estimation of products of lipid peroxidation include assays for conjugated dienes, malondaldehyde, hydroperoxides and fluorochromic products with features of Schiff bases (Buege, 1978). These methods all have one disadvantage in that they are measuring products of the normal pathway of lipid peroxidation as well as products of lipid oxidation and the thiobarbituric acid test which is often used is non-specific since compounds such as prostaglandins can interfere with this assay (Halliwell, 1984). It is now recognised when polyunsaturated lipids are exposed to free radicals in the presence of protein they can form non-peroxide diene-conjugated isomers. In humans 90% of the diene conjugates in human serum or plasma consist of the non-peroxide isomer of linoleic acid which can be measured and used as a marker of free radical activity (Iversen, 1985; Fink, 1985).

Free radicals have been implicated in the pathogenesis of many disease conditions including aging, inflammatory diseases, cancer, adult respiratory distress syndrome and tissue ischaemia and reperfusion (Dormandy, 1983; McCord, 1985). Their role in extending myocardial damage has been studied in animal models of myocardial reperfusion, largely by examining the protective effect of free radical scavengers (Werns, 1986). More recent animal studies

have attempted to measure free radicals in the ischaemic area of myocardium or from the venous effluent, but the results are conflicting, some groups suggesting increased free radical activity (Grill, 1987), while others have been unable to confirm this finding (Rosamond, 1987). The different results may well be related to the variety of experimental models used (Nayler, 1986). Despite the increasing literature in experimental models, little work has been done on free radical production in man in ischaemic heart disease or following acute myocardial infarction.

The aim of this study was to measure the non-peroxide diene conjugated isomer of linoleic acid (PL-9, 11-LA') as a marker of free radical activity in control subjects, patients with stable ischaemic heart disease and following acute myocardial infarction. In addition linoleic acid (PL-9, 12-LA) was measured and the molar ratio of PL-9, 11-LA'/PL-9, 12-LA calculated to allow for any change which may reflect the availability of the substrate, linoleic acid. The appearance of this marker of free radical activity was measured in plasma in the first 48 hours following myocardial infarction, by sequential blood sampling, and the effect of non-thrombolytic therapy compared with thrombolytic therapy. Results were correlated with white blood cell count, neutrophil count and creatine kinase.

PATIENTS AND METHODS

Patients and controls

The patients and controls studied comprised the same groups used for the measurement of plasma neutrophil elastase in chapter 6. These consisted of 35 healthy volunteers from the laboratory staff, 30 patients with stable ischaemic heart disease and 32 patients with acute myocardial infarction, 17 of whom were given intravenous thrombolytic therapy. Details of the groups are given in table 6(i) .

Protocols

The protocols were as described in chapter 6, blood also being taken for the non-peroxide diene conjugate of linoleic acid as a marker of free radical activity from all groups, sequential samples being taken at 8, 16, 24, 32, 40 and 48 hours in patients with acute myocardial infarction. Samples were centrifuged immediately and frozen at -20^{o}C until assayed.

METHODS

Full blood count and creatine kinase were measured as before.

PL-9, 11-LA', PL-9,12-1A'

Plasma was obtained from heparinised blood samples and stored at -20^{0}C until assayed. PL-9, 11-LA and PL-9, 12-LA were measured using high performance liquid chromatography (Gilson, 302; Villiers-de-Bel, France) with a variable wavelength ultraviolet detector set at 234 nm or 205 nm (Gilson, H M, Villiers-le-Bel, France). A Rheodyne injection valve (Corati, California, USA) was used with a 50 ul sample loop. A Spherisorb ODS2 250 x 4 mm column containing 5 um spherical particles (Hichrom, Reading, Berks, UK) was used with a mobile phase of acetonitrile/water/acetic acid 85:15:0.1 at a flow rate of 1.5 ml/min.

Plasma (0.5 ml) was mixed with 0.5 ml of a solution containing 0.1 mmol/l Tris pH 8.9, 1 mmol/l methanol and 5000 u/l phospholipase A_2. This mixture was incubated at 25^{o}C for 15 minutes after which 2 ml methanol containing 0.5% acetic acid and 50 mg/l beta-eleostearic acid [18:3 (9, 11, 13)] was added to precipitate the protein and add the internal standard. The preparation was centrifuged and 2 ml supernatant applied to a "Bond-Elut" column which had been washed twice with 2.5 ml propan-2-ol/acetonitrile (2:1) and twice with 2.5 ml of a wash solution of methanol/water/acetic acid (67:33:0.4). After the sample had been applied to the "Bond-Elut" column it was washed again with methanol/water/acetic acid and the sample

eluted with 1 ml propan-2-ol/acetonitrile (2:1). The eluate was directly injected into the HPLC using an auto-sampler (Perkin-Elmer ISS-IOI, Buckinghamshire, UK). The conjugated diene (PL-9, 11-LA') was measured at 234 mm and the non-conjugated fatty acid linoleic acid (PL-9, 12-LA) measured at 205 mm. The results are expressed as umol/l and the intra-assay coefficient of variation for the assays was less than 3.5%.

TRIS (Tris/hydroxymethyl) aminonethane and hydrochloride) buffer and phospholipase A_2 were supplied by Sigma, Dorset, UK. All solvents were supplied by Rathburn Chemicals Ltd, Lothian, UK.

Statistical analysis

This was performed as described in chapter 6. Results are expressed as median and range.

RESULTS

White blood count and creatine kinase (see results section, chapter 6).

ALL GROUPS

PL-9, 11-LA'

There was no significant difference in PL-9, 11-LA'

between normals (19.3 umol/l 7.5-32.9) and patients with stable ischaemic heart disease (19.8 umol/l 7.9-43.2). As a group the patients with myocardial infarction had significantly higher levels of PL-9, 11-LA' at all sample times 8-48 hours compared to controls, but only between 8 and 40 hrs compared to patients with ischaemic heart disease. Statistical results are summarised in tables 7(ii and iii). The individual results for patients with myocardial infarction are shown in figures 7c and 7d and mean data in 7e.

PL-9, 12-LA

There was no significant difference in PL-9, 12-LA between normals (390.5 umol/l, 281.0 - 634.4) and patients with stable ischaemic heart disease (352.7 umol/l, 214.2 - 586.7) or either group compared to the patients with acute myocardial infarction between 8 and 48 hours (406.4 umol/l, 240.8 - 584.6, 8 hour value).

Molar ratio PL-9, 11-LA'/PL-9, 12-LA

There was no significant difference in the molar ratio between normals (4.7%, 1.9-9.1) and patients with ischaemic heart disease (5.4%, 1.7-12.1). The molar ratio in patients with acute myocardial infarction was significantly elevated at sample times 8-48 hours compared to controls, but only 8-24 hours compared to patients with ischaemic heart disease (fig 7f).

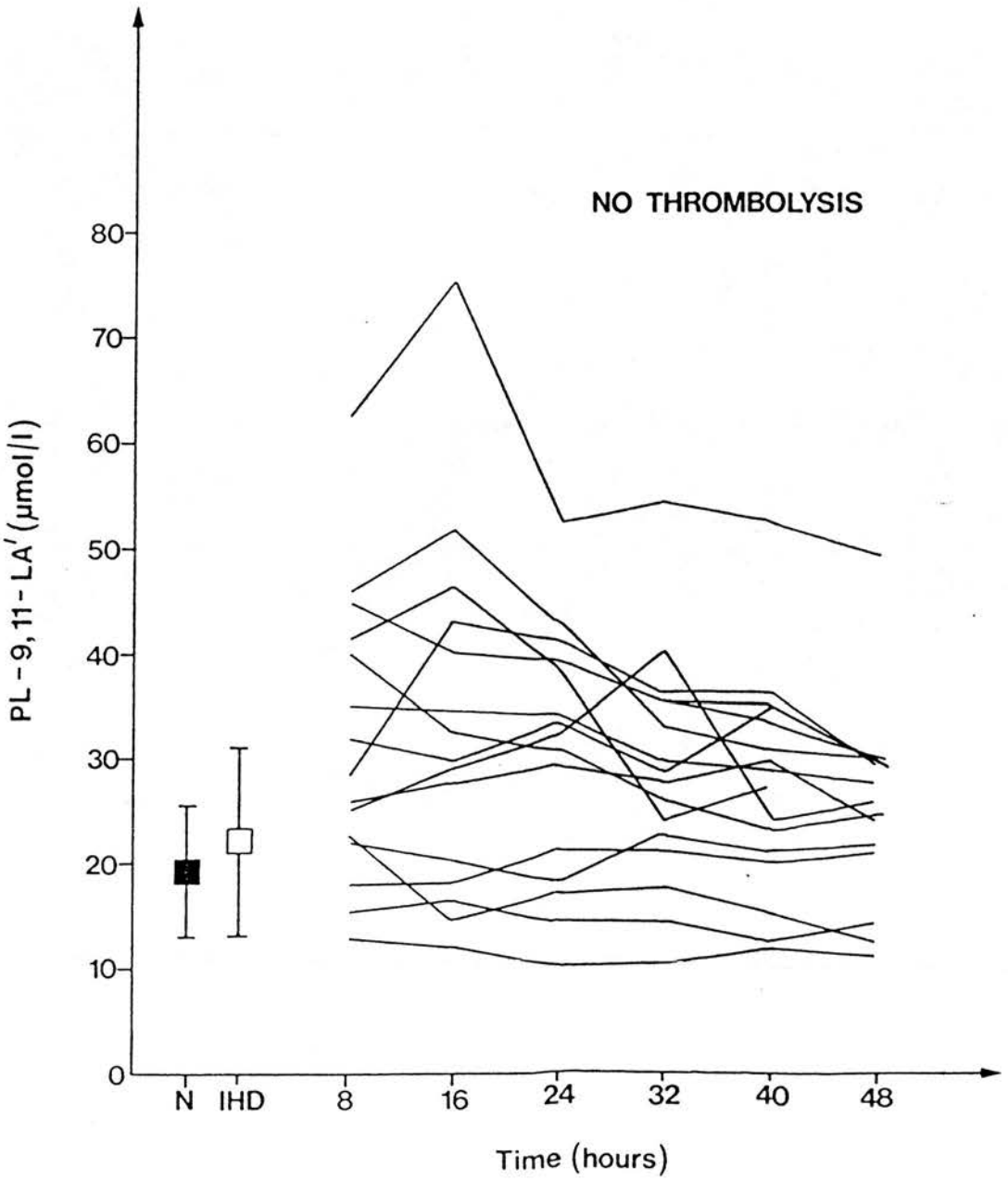


Fig 7c

Individual results for PL-9, 11-LA' between 8 and 48 hours in the 15 patients with acute anterior myocardial infarction who were not given thrombolytic therapy. Results for control subjects (N) and patients with ischaemic heart disease (IHD) are shown as mean ± 1SD for comparison.

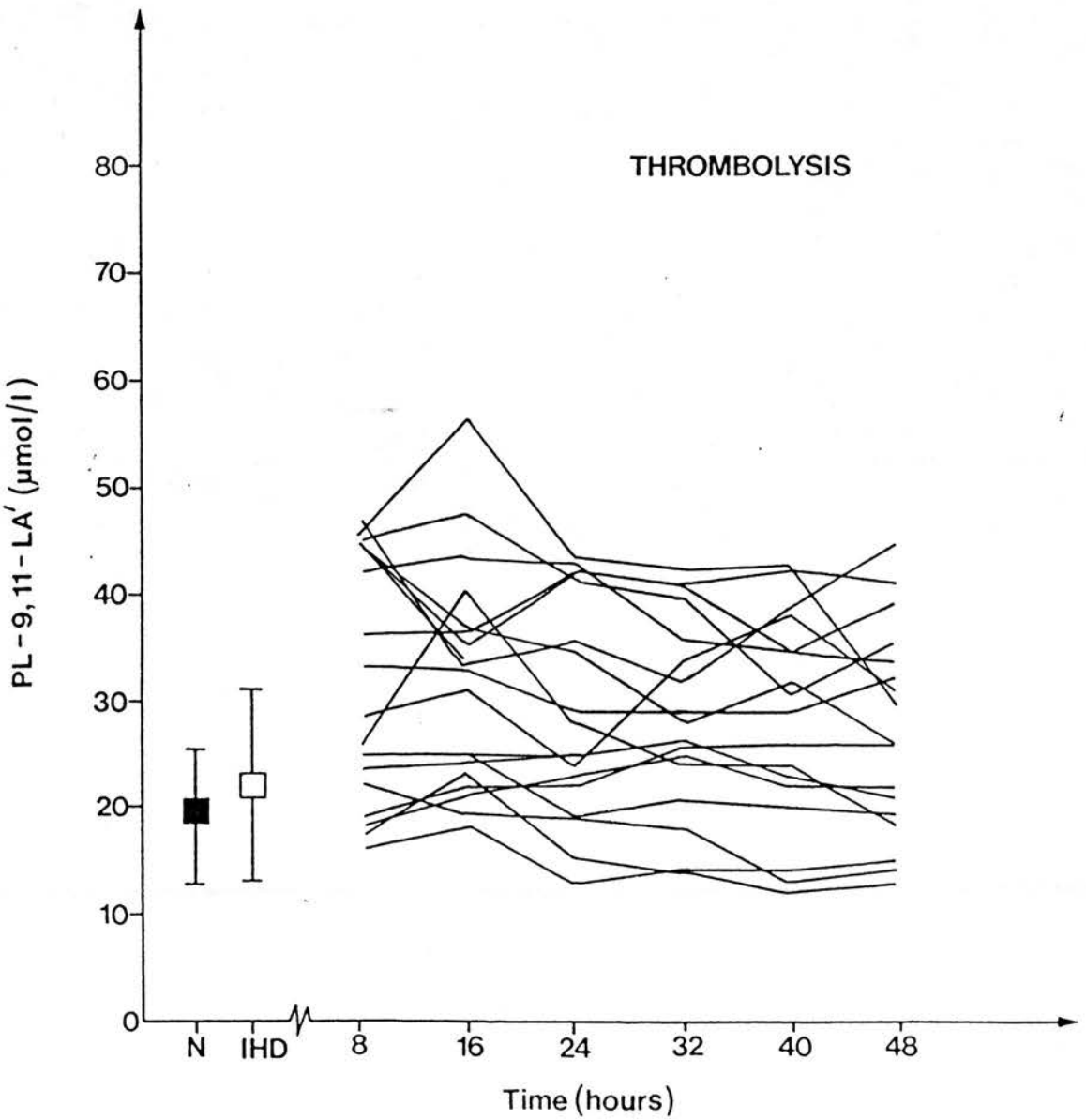


Fig 7d

Individual results for PL-9, 11-LA' between 8 and 48 hours in the 17 patients with acute myocardial infarction treated with thrombolytic therapy. Results for control subjects (N) and patients with ischaemic heart disease (IHD) are shown as mean ± 1SD for comparison.

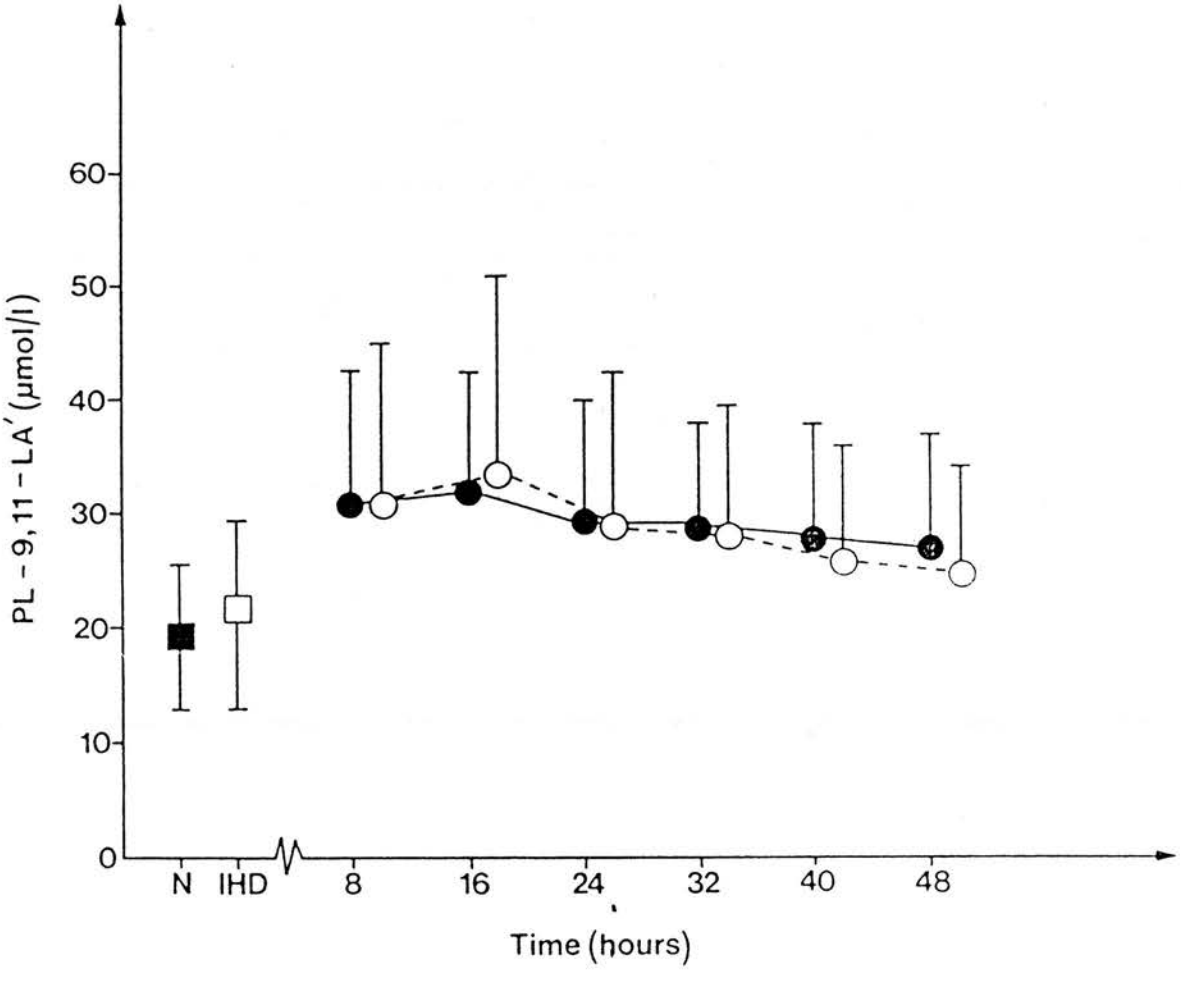


Fig 7e

Mean data (+ 1SD) for Pl-9, 11-LA' comparing treatment with (●) and without thrombolytic therapy (o). Levels are significantly increased at all times compared to controls, but there is no difference between treatment with or without thrombolytic drugs.

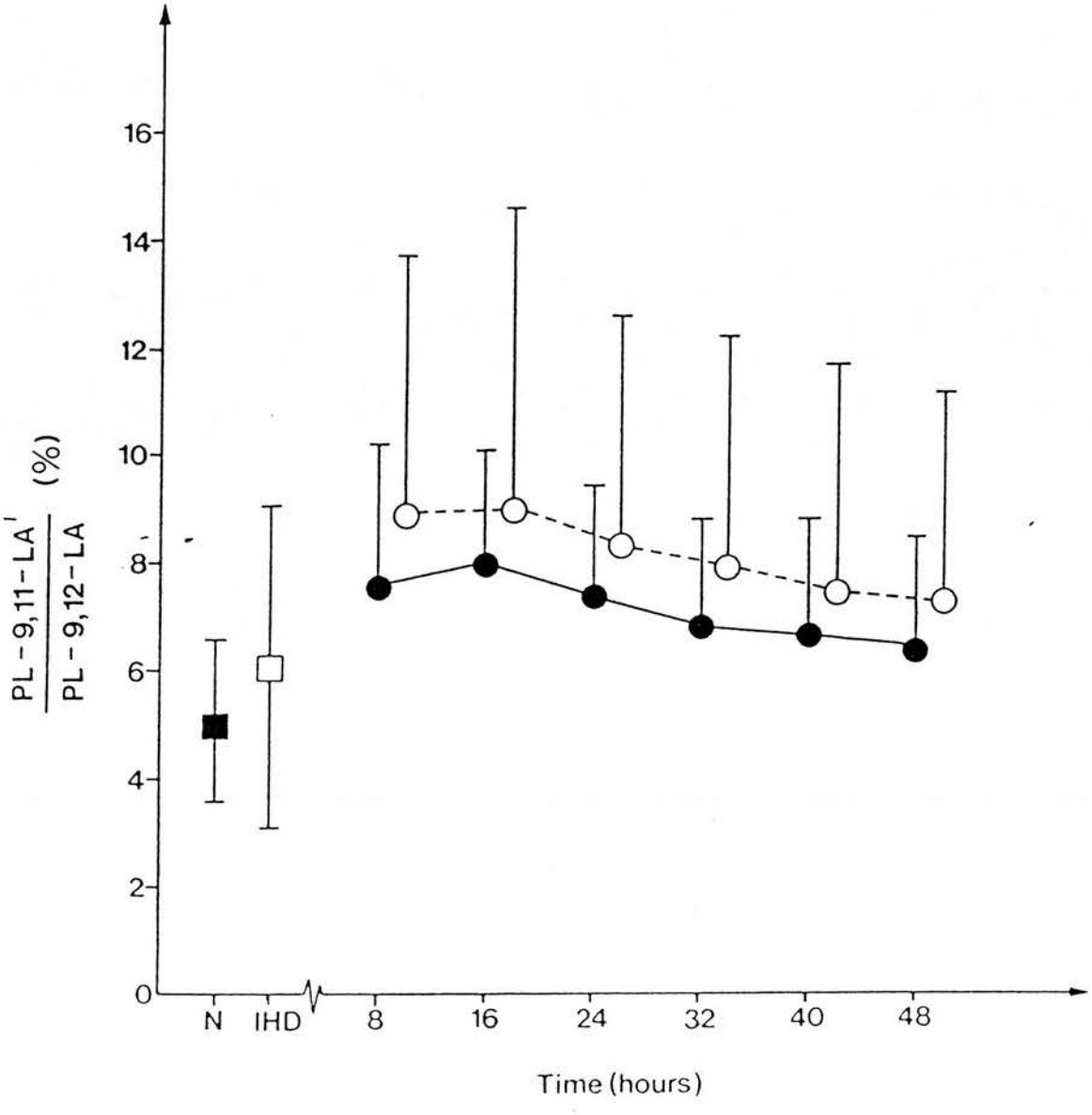


Fig 7f

Mean data (+ 1SD) for the molar ratio of PL-9, 11-LA'/PL-9, 12-LA comparing treatment with (●) and without thrombolytic therapy (o).

Comparison of patients with myocardial infarction treated with and without thrombolysis

PL-9, 11-LA', PL-9, 12-LA and the molar ratio PL-9, 11-LA'/PL-9, 12-LA

The median values of PL-9, 11-LA' and the molar ratio were highest in patients treated conventionally at 24 hours and at 16 hours for these treated with thrombolysis, but there was no significant difference between the two groups and measured data is shown diagrammatically in figs 7e and 7f. Similarly there was no difference in the substrate linoleic acid (PL-9, 12-LA) between the two groups over the 48 hours following myocardial infarction.

Correlations

There was no correlation between the peripheral white blood cell count or neutrophil count and PL-9, 11-LA' in the normal volunteers or the patients with acute myocardial infarction given thrombolysis. Correlations did exist for white blood cell count in patients with ischaemic heart disease ($r = 0.45$, $p < 0.02$) and the non-thrombolytic group of patients with myocardial infarction ($r = 0.63$, $p < 0.02$), but this was not true for control subjects or patients treated with thrombolysis. Further, there was no correlation between molar ratio and white blood cell count for any group. There was no correlation with PL-9, 11-LA' or the molar ratio and creatine kinase

in patients with acute myocardial infarction.

DISCUSSION

Free radicals have been implicated in myocardial injury following myocardial infarction, particularly following reperfusion (Werns, 1986). Exposure of cell membranes to oxygen radicals stimulates the process of "lipid peroxidation", probably better called lipid oxidation. The pathway of lipid peroxidation has been well studied and is often used as a marker of free radical activity and lipid peroxides have themselves been implicated in the pathogenesis of ischaemic injury in myocardial infarction (Meerson, 1982; Corr, 1984). The mechanisms proposed for this include: a) changes in the lipid microenvironment of membrane bound enzymes, receptors and ion channels which can both activate and inhibit the functional activity of such proteins; b) formation of new permeability channels secondary to membrane fragmentation; c) formation of cross-links between proteins and phospholipids of biomembranes, coupled with irreversible inactivation of the protein, and d) the oxidation of sulphydryl groups in the active sites of membrane bound enzymes with resulting loss of functional activity (Meerson, 1982). Whether lipid peroxides are themselves cytotoxic is a subject of debate, however, the products of this pathway are commonly used as markers of free radical activity (Buege, 1978).

Until recently most assays have measured products of lipid oxidation and peroxidation. However, it is now recognised that the main diene conjugate in human plasma is the non-peroxide isomer of linoleic acid (PL-9, 11-LA') which can be measured by HPLC and can be used as a marker of free radical activity (Iversen, 1985).

The results show that plasma levels of PL-9, 11-LA' and the molar ratio of PL-9, 11-LA'/PL-9, 12-LA, as markers of free radical activity are not significantly increased in patients with ischaemic heart disease compared to normal control subjects. Further, as the patients with ischaemic heart disease are older, this should accentuate any age related effects, as ageing is thought to be associated with greater free radical activity (Dormandy, 1983; Halliwell, 1985). However, the patients with stable ischaemic heart disease are of similar age to those with acute myocardial infarction allowing a comparison between these groups. Patients with acute myocardial infarction have increased levels of both PL-9, 11-LA' and the molar ratio of PL-9, 11-LA'/PL-9, 12-LA in the first 32 hours after the onset of symptoms, compared with control subjects and patients with ischaemic heart disease.

In patients with acute myocardial infarction, the levels of PL-9, 11-LA' and the molar ratio were both increased

whether treated with or without thrombolytic therapy with no statistical difference evident between the groups. The molar ratio was slightly, but not significantly, lower in the patients who received thrombolytic therapy. These results suggest that reperfusion, following lysis of coronary thrombus, is not associated with greater lipid oxidation and hence by inference increased free radical activity in the period 8-48 hours following myocardial infarction. These results do not exclude a very early and transient increase in free radical activity following myocardial reperfusion suggested by some studies (Garlick, 1987), but do imply that reperfusion is not associated with continued or delayed free radical activity in man. Perhaps this reflects the beneficial effects of reperfusing the heart with blood, rather than the cell free perfusate often used in animal experiments, as free radical damage may be reduced because of the presence of catalase, the free radical scavenger, present in red blood cells (Jolly, 1984; Agar, 1986). The results do not necessarily conflict with the experimental evidence which has shown that pre-treatment of animals with free radical scavengers has a beneficial effect on myocardial salvage which by inference suggests free radical mediated myocardial injury (Ambrosio, 1986; Myers, 1985). Free radical scavengers, such as superoxide dismutase, may also exert their beneficial by effect by preventing the generation of a superoxide dependent neutrophil

chemotactic factor (McCord, 1982). Thus, by preventing the accumulation of neutrophils at the potential site of inflammation, not only will superoxide dismutase prevent the tissue from direct attack by O_2^-, but indirectly this will prevent tissue injury by the additional mechanisms available to activated neutrophils, such as the release of potent proteolytic enzymes.

If neutrophils were the major source of oxygen free radical activity, associated with secondary myocardial injury, then one might expect a delayed increase in PL-9, 11-LA' coincident with neutrophil migration into the area of myocardial injury. The late increase in neutrophil elastase, seen in patients with myocardial infarction treated without thrombolytic therapy, is consistent with neutrophil degranulation within the area of myocardial ischaemia, but a secondary increase in PL-9, 11-LA' was not seen in either of the groups of patients with myocardial infarction. Also, although a correlation between the peripheral white cell count and PL-9, 11-LA' was found in patients with stable ischaemic heart disease and patients with myocardial infarction treated conventionally, no correlation was found in normals or the patients given thrombolysis. Further, the molar ratio, which takes account of the available substrate, shows no correlation with neutrophil count for any of the groups. Therefore, the results do not show a consistent

association between the degree of lipid oxidation and neutrophils, therefore, the source or sources of increased free radical generation in myocardial infarction is still not fully established. As there is no correlation between creatine kinase and PL-9, 11-LA' it seems unlikely that PL-9, 11-LA' is merely a marker of ischaemic cell injury (Halliwell, 1984) as it does not appear to be influenced by infarct size.

In other species the diene conjugated non-peroxide isomer of linoleic acid (9, 11-LA') may be generated from other sources such as liver microsomes and can also reflect dietary intake following bacterial dehydrogenation of linoleic acid (Thompson, 1985). In this study, it is unlikely that dietary sources could affect the results as serial sampling over 48 hours showed that the levels of PL-9, 11-LA' were initially high and fell with time, whereas the levels of PL-9, 12-LA which may also be affected by diet (Wood, 1987) did not alter significantly with time. Further, changes in PL-9, 11-LA' which could relate to the availability of the substrate PL-9, 12-LA are incorporated by calculating the molar ratio which is similarly increased following myocardial infarction and follows a similar time course to appearance of PL-9, 11-LA'.

The results suggest that there is increased free radical

activity, as measured by products of lipid oxidation, in patients following myocardial infarction but not in patients with stable ischaemic heart disease. The increase in free radical activity is greatest in the first 16 hours following myocardial infarction, but does not merely reflect infarct size and shows no secondary or delayed increase simultaneous with the acute inflammatory infiltrate. Thrombolytic therapy is not associated with greater delayed free radical production, compared to treatment without thrombolysis, though this has been postulated as a mechanism of reperfusion injury. The source of increased free radical production following myocardial infarction is therefore not yet established and though the neutrophil may be involved, this is likely to be only one of several mechanisms.

CHAPTER 8

SUMMARY

8.1 THE SEPARATION, RADIOLABELLING AND SUBSEQUENT IN-VIVO BEHAVIOUR OF RADIOLABELLED AUTOLOGOUS NEUTROPHILS

1. Mono-poly resolving medium, a Ficoll-hypaque based cell separation medium, is a reliable method for isolating neutrophils from whole blood. It is a rapid method which allows separation of neutrophils in a single step, thus reducing the handling of the cells with the associated risk of mechanical damage. Separation was not possible in patients with chronic obstructive lung disease who represented most of the 10% failure rate.

2. The neutrophil recovery using this technique is good and compares favourably with other methods for separating neutrophils from blood. Lymphocyte contamination is negligible and red cell contamination is small and insufficient to interfere with efficient radiolabelling of the neutrophils.

3. Autologous neutrophils separated using mono-poly resolving medium can be radiolabelled efficiently

with Indium-111-oxine and when injected into patients show normal in vivo behaviour. Thus, after bolus injection of the labelled neutrophils there is rapid clearance from the pulmonary circulation, with no continued accumulation of neutrophils within the liver to suggest removal of damaged cells by the hepatic reticulo-endothelial system. This is compatible with the cells functioning normally within the circulation and as the cells also successfully migrate to sites of infection or inflammation this indicates that they can respond to chemotactic stimuli.

8.2 IMAGING THE INFLAMMATORY RESPONSE TO ACUTE MYOCARDIAL INFARCTION USING INDIUM-111 LABELLED AUTOLOGOUS NEUTROPHILS

1. The acute inflammatory cellular infiltrate, within infarcted myocardium, can be imaged in man using Indium-111 labelled autologous neutrophils.

2. The principal factor which determines whether labelled neutrophils can be detected at the site of myocardial infarction is the interval between the onset of symptoms and injection of the labelled neutrophils. Overall 77% of patients with acute myocardial infarction in this study had positive

Indium images. However, all patients injected within 18 hours had positive images.

3. No other factor, including peripheral leucocytosis, estimates of infarct size or drug therapy influenced the likelihood of obtaining positive uptake of labelled neutrophils in damaged myocardium.

4. Single photon emission computed tomography improved the spatial separation of myocardial uptake of labelled neutrophils from uptake in the adjacent organs, namely spleen, liver and bone and hence reduced the number of false negative results which would have been obtained with conventional planar imaging alone.

5. The combination of single photon emission computed tomography with simultaneous radiolabelled neutrophil and blood pool imaging increased the certainty that uptake was present within the region of myocardium and reduced the possibility of false positive results.

6. The uptake of Indium-111 labelled neutrophils at the site of myocardial infarction is not a technique to be used to confirm myocardial infarction.

8.3 A COMPARISON OF THE INFLAMMATORY RESPONSE AND INFARCT SIZE, ASSESSED BY IMAGING TECHNIQUES, IN PATIENTS WITH ACUTE MYOCARDIAL INFARCTION TREATED WITH AND WITHOUT THROMBOLYTIC THERAPY

1. The extent of Indium-111 neutrophil uptake within myocardium is significantly less compared to the extent of myocardial damage assessed by Technetium-99m pyrophosphate in patients with acute anterior myocardial infarction.

2. There is no correlation between the inflammatory response assessed by labelled neutrophils and the size of myocardial infarction assessed by pyrophosphate imaging, peak creatine kinase, or residual left ventricular function.

3. Management of acute myocardial infarction, with thrombolytic therapy, appears to reduce the extent of neutrophil uptake relative to infarct size, when compared with patients treated without thrombolytic therapy.

4. Despite early injection of labelled neutrophils myocardial uptake of Indium could not be detected in two of the seventeen patients treated with thrombolytic therapy. These may represent true

failures, but equally may be the result of successful reperfusion as thrombolysis appears to reduce the extent of the acute inflammatory infiltrate in acute myocardial infarction.

5. The estimation of infarct size by Technetium-99m pyrophosphate correlates with the two other indirect estimates of infarct size measured, namely peak creatine kinase and residual left ventricular ejection fraction.

8.4 PLASMA NEUTROPHIL ELASTASE IN ISCHAEMIC HEART DISEASE AND PATIENTS WITH ACUTE MYOCARDIAL INFARCTION TREATED WITH AND WITHOUT THROMBOLYTIC THERAPY

1. As a group patients with stable ischaemic heart disease have a slightly but significantly increased level of plasma neutrophil elastase. As few patients admitted to angina on the day of blood sampling, ischaemic pain does not appear to influence the levels of elastase. Whether the elevated levels of plasma neutrophil elastase reflect neutrophil activation in close proximity to the endothelium with possible endothelial injury or merely reflect neutrophil endothelial interaction at sites of pre-existing ahteroma, remains to be

established.

2. Plasma levels of neutrophil elastase are significantly increased in the 48 hours following acute myocardial infarction, compared with control subjects or patients with ischaemic heart disease. This increase does not correlate with the peripheral neutrophil count at the time of sampling.

3. The patterns of appearance of neutrophil elastase in plasma differs in patients treated without thrombolytic therapy compared to patients who were given thrombolytic agents. Patients managed conventionally showed only a moderate increase in neutrophil elastase over the first 24 hours, but had a late peak at 40 hours. This increase occurs when neutrophils are present within the injured area of myocardium and may reflect local myocardial release with the potential for secondary myocardial injury. In contrast, patients given thrombolytic therapy had high initial values of neutrophil elastase at 8 hours which tended to fall over the next 48 hours. It is unlikely that many neutrophils will have migrated into the damaged area of myocardium as early as 8 hours, therefore elevated levels at this stage may reflect intravascular neutrophil activation following contact with damaged

endothelium or release from neutrophils trapped within the thrombus.

4. None of the patients in this study underwent emergency coronary angiography therefore the true rates of spontaneous reperfusion or unsuccessful thrombolysis are not known which makes interpretation of the different pattern of results in patients treated with and without thrombolysis difficult.

5. A correlation exists between plasma neutrophil elastase and creatine kinase which suggests that the release of neutrophil elastase is in some way related to the extent of myocardial injury and may therefore be a mechanism of secondary myocardial injury.

8.5 LIPID OXIDATION PRODUCTS AS A MARKER OF FREE RADICAL ACTIVITY IN PATIENTS WITH STABLE ISCHAEMIC HEART DISEASE AND ACUTE MYOCARDIAL INFARCTION TREATED WITH AND WITHOUT THROMBOLYTIC THERAPY

1. There is no difference in the plasma levels of the non-peroxide diene conjugated isomer of linoleic (PL-9,11-LA') acid or the molar ratio of this isomer with the substrate linoleic acid in patients with

stable ischaemic heart disease compared to control subjects. This suggests that there is no significant increase in lipid oxidation and hence free radical production in patients with stable ischaemic heart disease.

2. In patients with acute myocardial infarction free radical activity assessed by this method was significantly greater than patients with stable ischaemic heart disease or control subjects. The levels were higher in the first 24 hours and fell towards normal values in the period 24 - 48 hours following the onset of symptoms.

3. The levels of linoleic acid were no different in control subjects, patients with ischaemic heart disease or acute myocardial infarction. It is therefore unlikely that dietary intake of unsaturated fatty acids has influenced the results.

4. Although the levels were increased following acute myocardial infarction, there was no difference in the non-peroxide diene conjugate of linoleic acid or the molar ratio in patients treated with or without thrombolytic therapy. The molar ratio was slightly, but not signficantly, less in patients treated by thrombolysis compared to patients treated

conventionally. These results do not suggest that successful reperfusion is associated with greater free radical production, though this is a postulated mechanism of reperfusion injury, following successful thrombolysis.

5. It has been suggested that increased lipid oxidation products merely reflect the extent of tissue injury (Halliwell, 1984). However, the levels of the non-peroxide diene conjugate of linoleic acid or the molar ratio do not correlate with infarct size assessed by creatine kinase in this study. This suggests that PL-9,11-LA' does not purely reflect cell membrane damage and is consistent with increased free radical activity.

6. The source of free radical production, assessed by lipid oxidation, is not established. The correlation of PL-9,11-LA' with neutrophil count was not consistent and furthermore was not found with the molar ratio in either patients with stable ischaemic heart disease or acute myocardial infarction. Also as there was no late increase in plasma PL-9,11-LA' coincident with neutrophil infiltration of damaged myocardium, this would suggest that neutrophils are not the major source of free radical activity.

CHAPTER 9

GENERAL DISCUSSION AND FUTURE DIRECTIONS

The purpose of this thesis was to examine the potential role of the acute inflammatory response, with a particular emphasis on the importance of the neutrophil, in secondary myocardial injury following myocardial infarction and to also study the effects of thrombolytic therapy on this response. This was undertaken in three ways; (1) by imaging the neutrophil uptake at the site of myocardial damage using radiolabelled autologous neutrophils, (2) sequential measurements of plasma neutrophil elastase as a measure of neutrophil activation, and (3) sequential measurements of the non-peroxide diene conjugate of linoleic acid in plasma as a marker of free radical activity.

The method described for the separation and radiolabelling of autologous neutrophils from whole blood in a single step proved to be rapid and efficient in patients with acute myocardial infarction. These neutrophils demonstrated normal in vivo behaviour with rapid pulmonary clearance and migration to sites of infection and inflammation suggesting that they were functionally active. Providing patients with myocardial infarction were injected with the radiolabelled cells within 18 hours

of the onset of symptoms the uptake of neutrophils at the site of myocardial damage could be reliably imaged. This suggests that the main chemotactic stimuli for neutrophil migration occur early and probably do not persist for greater than 24 hours. Complement components in particular C3a and C5a are known to be important in neutrophil migration to the area of myocardial damage (Pinckard, 1975; Hartmann, 1977). The advent of monoclonal antibody based assays to these components should make it possible to determine the extent and duration of complement activation following myocardial infarction. Many other inflammatory mediators may be involved, particularly the leukotrienes, interleukin and platelet activating factor and again the introduction of reliable assay methods should allow improved understanding of the mechanisms controlling the inflammatory response to myocardial injury.

Single photon emission computed tomography (SPECT) is a significant advance in myocardial imaging: SPECT allows spatial separation of myocardial uptake ^{111}In-neutrophils and ^{99m}Tc-pyrophosphate of from uptake in surrounding organs including bone. It also allows quantification of radionuclide uptake by counting the number of voxels (volume cell elements) with uptake of ^{111}In or ^{99m}Tc present within the region of myocardium. In general, using this method, the inflammatory infiltrate assessed by

^{111}In-neutrophil imaging was less extensive than the actual size of infarct assessed by pyrophosphate imaging. Further, the patients who were treated with thrombolytic therapy appeared to have a less marked neutrophil infiltrate for a given infarct size compared to patients who did not receive thrombolytic therapy. Previous observations suggested that successful reperfusion was associated with an early and increased neutrophil infiltrate (Sommers, 1964). However, these studies may be describing an early and transient component of the normal vascular phase of the inflammatory response to tissue injury. As successful reperfusion of an occluded vessel will produce earlier neutrophil influx and margination with some neutrophil extravascular migration but does not necessarily imply the inflammatory response is amplified, which is the common interpretation. It was not possible to address the question of the progression of the neutrophil infiltration in this study because of the difficulty in moving patients to a gamma camera in the early hours of their infarct. Although accurate quantification of the inflammatory infiltrate could not be studied, it should be possible to assess the course of this response using a mobile gamma camera with sequential imaging of the patients in the coronary care unit, providing patients could be injected with autologous neutrophils in the first few hours of their acute infarct. A comparison could then be made between patients treated

with and without thrombolytic therapy on the rate and extent of development of uptake of autologous neutrophils at the site of myocardial damage.

This study did not show a significant reduction in infarct size, estimated by peak creatine kinase, myocardial uptake of ^{99m}Tc-pyrophosphate or residual left ventricular function, in patients treated with thrombolytic therapy compared to those treated without thrombolytic therapy. This almost certainly reflects the size of the study group as we know from large multicentre trials that thrombolysis does improve mortality (Gissi, 1987; ISIS-2, 1988) and preserve myocardial function (Serruys, 1986). Despite similar infarct sizes, the neutrophil uptake was less in patients treated with thrombolytic agents, suggesting that some of the beneficial effects of thrombolysis may relate to reduction of the inflammatory response and associated secondary myocardial injury. If this is the case it probably would not be beneficial and perhaps even harmful to further reduce the inflammatory response in these patients because of the essential part the inflammatory process plays in normal healing. However, downregulation of the inflammatory response may be a mechanism for improving myocardial salvage in patients not eligible for thrombolytic therapy.

Corticosteroids are known to suppress inflammation, and

two studies in which an early but large doses of corticosteroids have been given suggest this can reduce infarct size (Morrison, 1976; Hammerman, 1983) but these results have been overshadowed by the potential deleterious effects on scar healing shown with the use of higher multidose therapy (Roberts, 1976; Kloner, 1978). The effects on scar thinning with the risk of ventricular aneurysm and rupture probably reflects the catabolic effects of steroids on collagen metabolism, rather than the anti-inflammatory effects, hence the potential benefit of a single early dose of corticosteroid following myocardial infarction. Although it would not be ethical to compare prolonged steroid therapy with early single dose steroid therapy it would, on the available evidence, be possible to compare early administration of high dose corticosteroids with no corticosteroid on infarct size, neutrophil uptake and residual ventricular function following acute myocardial infarction using the methods described in this thesis.

To further determine whether the neutrophil is an important factor in secondary myocardial injury drugs which have a more specific effect on neutrophil function could be used as experimental tools; for example some of the non-steroidal anti-inflammatory agents. Ibuprofen has been shown to reduce the infarct size in animals (Romson, 1982) and other workers have suggested that this

is a direct effect of ibuprofen on neutrophils rather than the effect of the drugs on prostaglandin metabolism (Rampart, 1986). Similarly, the potential role of superoxide dismutase in preventing production of a free radical derived chemotactic factor could also be examined. Such studies could again be undertaken using radiolabelled neutrophils and sequential gamma camera imaging in patients with myocardial infarction treated with drugs such as ibuprofen or superoxide dismutase.

Plasma neutrophil elastase is a marker of neutrophil activation and the release of lysosomal enzymes from the primary or azurophilic granules requires major stimulation of the cell such as phagocytosis or cell death. Proteolytic enzymes, in particular neutrophil elastase, have been implicated in the pathogenesis of a number of disease conditions (Malech, 1987) including myocardial infarction. It is also known that this enzyme can directly injure endothelial cells in culture and hence may be involved in vascular injury. It is therefore interesting that as a group patients with documented ischaemic heart disease have mildly elevated levels of this enzyme. Whether this represents mechanical damage to neutrophils secondary to pre-existing atheroma rather than truly reflecting a pathogenetic mechanism is unclear. The role of elastase in endothelial injury or atheroma could perhaps be further investigated by using specific

elastase antibody and immunoperoxidase techniques in histopathological sections of coronary vessels obtained at post-mortem or from heart transplant recipients. If increased levels were found in areas of atheroma compared to normal areas this would support the suggestion that elastase contributes to vascular disease (Robert, 1984).

In patients with acute myocardial infarction the levels of human neutrophil elastase are significantly increased in the first 48 hours following myocardial infarction compared to normal subjects or patients with ischaemic heart disease. This may be thought to be a normal response to stress. However, this is unlikely as we have shown that plasma neutrophil elastase does not correlate with the peripheral neutrophil count which itself increases as part of the normal stress response. The pattern of evolution of neutrophil elastase also differs in patients with myocardial infarction treated with and without thrombolytic therapy while presumably these patients are exposed to similar degrees of stress. The high initial levels of neutrophil elastase in patients treated with thrombolytic agents suggest intravascular release whereas the later increase in plasma neutrophil elastase seen in patients treated without thrombolysis is consistent with enzyme release from activated neutrophils present within the area of myocardial necrosis. This hypothesis could be further investigated by measuring

neutrophil elastase and creatine kinase in the venous effluent from the coronary sinus in patients who require haemodynamic monitoring following myocardial infarction. It would also be interesting to examine other neutrophil products, such as lactoferrin, to confirm the sequence of events seen with elastase and also to relate this to the presence of inflammatory mediators such as components of the complement system (Hartmann, 1977).

Free radicals have also been implicated in secondary myocardial injury (Werns, 1986). The diene conjugated non-peroxide isomer of linoelic acid is a product of lipid oxidation and is thought to measure free radical activity in man (Iversen, 1985). This is the major conjugated diene in human plasma and is a product of lipid oxidation, which is felt to be a more useful marker of free radical activity than other methods which measure the end products of the normal lipid peroxidation pathway and hence assess both products of oxidation and peroxidation. Similarly, some assays of lipid peroxidation such as thiobarbituric acid are non-specific (Halliwell, 1984) as they also detect prostaglandins and glycosylated proteins.

The results show that the plasma levels of non-peroxide diene conjugated isomer of linoelic acid is similar in patients with stable ischaemic heart disease and normal subjects. This suggests that lipid oxidation and hence

free radical activity is not necessarily increased in patients with atheroma in the absence of tissue ischaemia. In contrast levels are high in patients with acute myocardial infarction, peak levels occurring at approximately 16 hours after the onset of symptoms in patients treated with or without thrombolysis. As there was no difference in the levels of PL-9, 11-LA' between between patients treated with or without thrombolysis this does not support the experimental evidence which suggests that increased free radical production is a major component of reperfusion injury. Also in patients who did not receive thrombolytic therapy, there was no late increase in levels of PL-9, 11-LA', similar to that found with plasma neutrophil elastase (figs 9a and b). Perhaps, therefore, the release of proteolytic enzyme is the more important mechanism of secondary tissue injury.

There has been some controversy in the literature over the use of PL-9, 11-LA' as a marker of free radical activity. Part of this has arisen because it was felt to be a marker of cervical cancer (unrelated to free radical production) (Singer, 1987). However, this is now thought to be due to bacterial colonisation (Green, 1988). Similarly it has been suggested that increased levels in man may represent bacterial dehydrogenation of linoleic acid in the gastrointestinal lumen with subsequent absorption (Thompson, 1985). This suggestion would not explain the

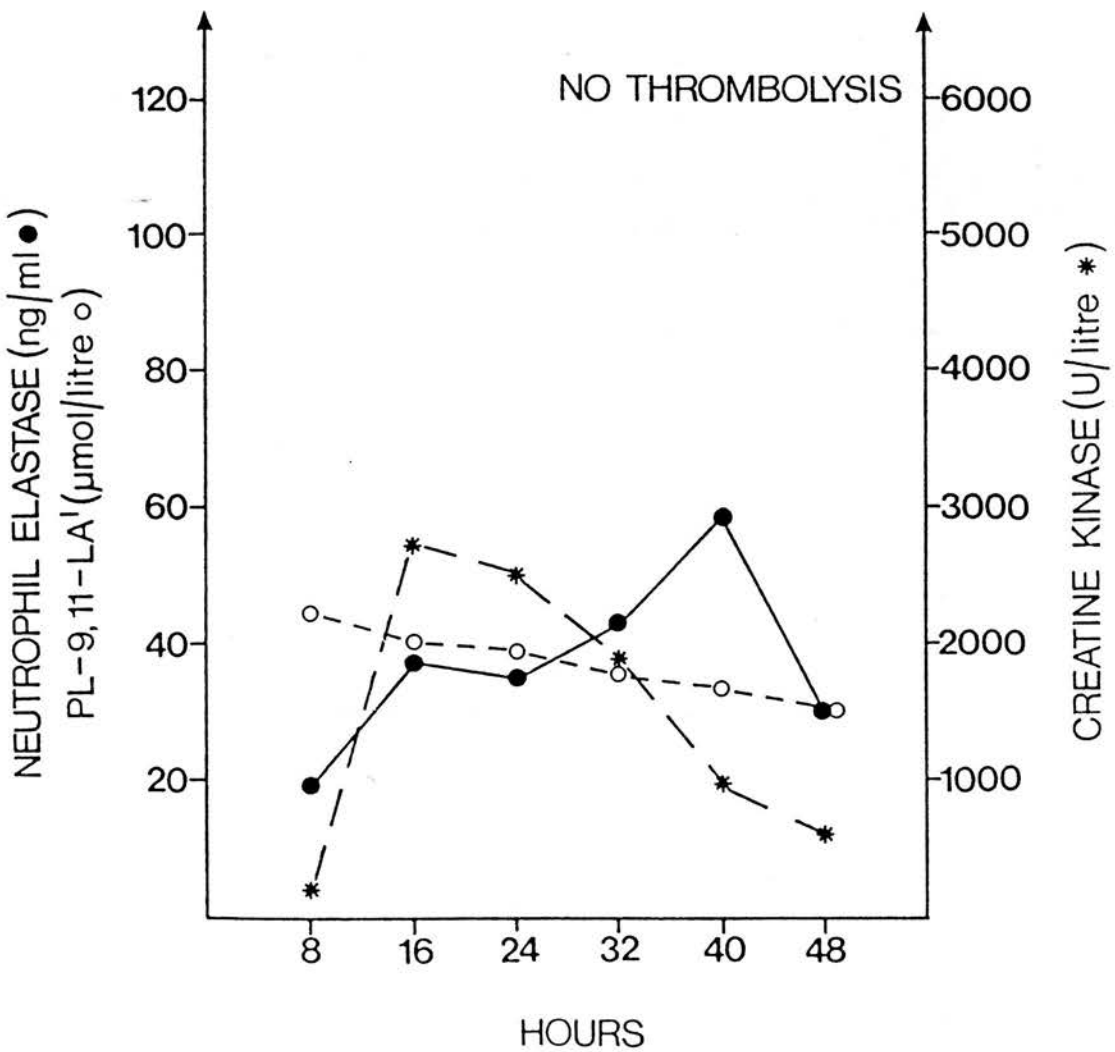


Fig 9a

Changes in plasma creatine kinase, plasma neutrophil elastase and plasma PL-9,11-LA' in patient not treated with thrombolytic therapy.

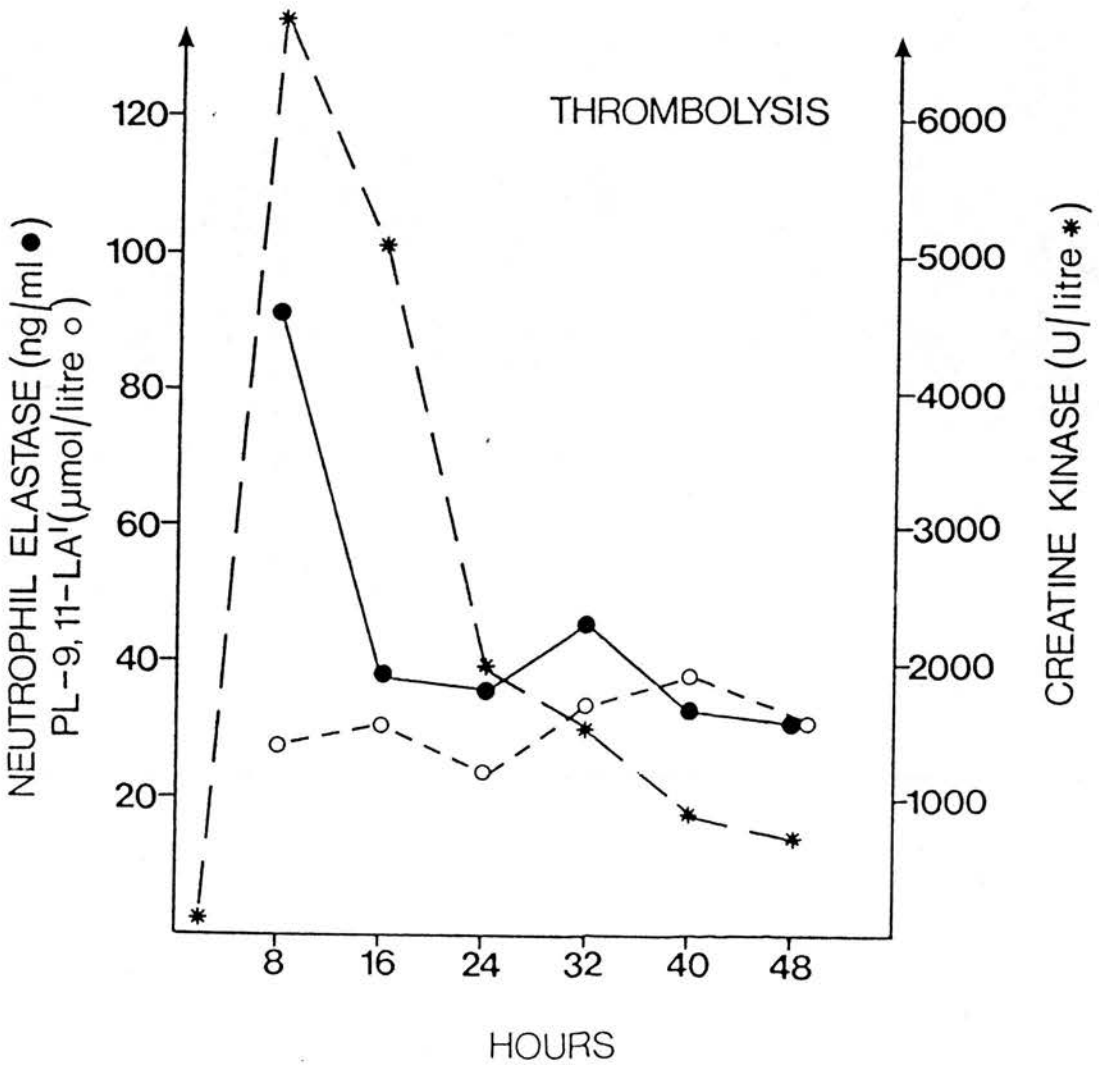


Fig 9b

Changes in plasma creatine kinase, plasma neutrophil elastase and plasma PL-9,11-LA' in patient treated with intravenous thrombolytic therapy.

differences between patients with ischaemic heart disease or the acute increase seen in patients with myocardial infarction. It should also be remembered that PL-9, 11-LA' is the major diene conjugate in human plasma and that measurement of total diene conjugates is still considered to reflect increased free radical production (Lunec, 1981). Finally, the levels of linoleic acid which can also be altered by diet did not differ with time in patients with myocardial infarction. The answer to the role of free radicals in myocardial ischaemia will probably only be answered using techniques to measure free radicals directly such as electron spin resonance (Mason, 1984).

By producing alterations in cell membranes, lipid peroxides have themselves been implicated in cell damage (Meerson, 1984) and it is possible that the non-peroxide diene conjugated isomer of linoleic acid not only reflects free radical activity, but also may itself mediate cell damage. Recent work has confirmed the presence of another metabolite of linoleic acid (linoleate epoxide), named leukotoxin, which is generated by neutrophils and may be the result of a monoxygenation of linoleic acid by free radicals (Oliw, 1981). Leukotoxin has now been shown to be a potent mediator of pulmonary damage (Ozawa, 1988) and hence could also have a role in secondary myocardial injury. By measuring leukotoxin and the non-

peroxide diene conjugated isomer of linoleic acid simultaneously further information about the source of oxygen free radicals and mechanism of secondary myocardial injury may be gained. These measurements could also be combined with neutrophil elastase to assess the potential therapeutic effects of drugs such as ibuprofen and free radical scavengers on neutrophil function in vivo, following myocardial infarction. Interestingly, it has also been recently suggested that the angiotensin converting enzyme inhibitor captopril, because of its sulphydril group, may also be a free radical scavenger (Westlin, 1988).

Using the techniques described in this thesis it would be interesting to study other aspects of ischaemic heart disease. For instance, does the neutrophil have a role in unstable angina, perhaps through neutrophil margination, contributing to microvascular occlusion, with or without neutrophil activation. It would also be interesting to study the effect of balloon angioplasty of a coronary vessel on neutrophil activation and free radical production, as this technique must produce endothelial damage. If there is a significant endothelial/neutrophil interaction it should be possible to detect this by measuring neutrophil elastase, the non-peroxide diene conjugate of linoleic acid and perhaps other inflammatory mediators in the coronary sinus

effluent or peripheral blood.

The studies outlined in this thesis show that there is a significant acute inflammatory response to mycoardial infarction, which can be imaged using radiolabelled autologous neutrophils and that this is associated with evidence of neutrophil activation. There is also evidence of increased free radical activity, though the source of free radical generation remains uncertain. They suggest that these mechanisms may be important in secondary myocardial injury. However, the results do not support, experimental evidence, which suggests that successful reperfusion is associated with an enhanced inflammatory response with the associated potential to produce further damage to already injured myocytes.

Ischaemic heart disease remains the commonest cause of death in industrialised countries often affecting the younger age group. Athough the therapeutic benefits of thrombolytic therapy in terms of mortality and improved myocardial salvage are now established (GISSI-2, ISIS 2), this therapy is not suitable for all patients. Estimates suggest that only 14 to 50% of patients fulfil the current criteria for thrombolysis (de Bono, 1987). It therefore seems important to pursue other methods of improving myocardial salvage as this may still significantly contribute to reduced mortality. Experimental evidence

from this and earlier work suggests that the neutrophil has a role in secondary myocardial injury and it is interesting to speculate that some of the beneficial effects of thrombolysis might be related to reducing the intensity of the acute inflammatory infiltrate, at the site of myocardial damage. Potential methods to further study the importance of the neutrophil in myocardial infarction appear indicated some of which are outlined in this discussion.

APPENDIX I

The work for this dissertation was carried out entirely within the Department of Medicine, University of Edinburgh, Royal Infirmary, Edinburgh under the supervision of Dr A L Muir, Reader, Department of Medicine. The practical work was performed by myself or Miss Melanie Jackson. A second observer was used in the detection of myocardial uptake of the radioisotopes and Dr J J Nicoll assisted in the calculation of volume of uptake. My appointment during the time of preparation of this thesis was as Lecturer in General Medicine, Department of Medicine, University of Edinburgh, Royal Infirmary, Edinburgh and since November, 1988 as Senior Registrar in Thoracic Medicine, Central Middlesex Hospital, London.

Part of the work presented has appeared in published form.

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Muir A L, Bell D , Jackson M, Nicoll J J, Millar A M. The use of ^{111}In-labelled autologous neutrophils in imaging myocardial infarction. Nucl Med Comm 1988; 9: 707-711.

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TABLE 3(i)

PATIENT DETAILS, NEUTROPHIL RECOVERY, LABELLING EFFICIENCY AND SCAN OUTCOME

PATIENT	SEX	AGE	REASON FOR SCAN	NEUTROPHIL RECOVERY (%)	NEUTROPHIL RECOVERY ($x10^7$)	LABELLING (%)	SCAN Pos/Neg
1. HW	M	72	Effusion ? empyema	65	65.0	87	Neg
2. HD	F	81	Pneumonia	64	61.4	84	Neg
3. GA	M	19	? intra-abdominal abscess	27	12.4	55	Neg
4. CA	F	73	Pseudomembranous colitis	19	22.6	74	Pos
5. RM	M	46	? intra-abdominal abscess	19	10.1	74	Pos
6. DC	M	49	? intra-abdominal abscess	57	29.4	76	Pos
7. GM	M	78	? renal abscess (kinetic study)	64	20.7	77	Pos
8. RL	M	57	Pyrexial ? cause	75	39.4	70	Pos
9. VV	F	NK	? brain abscess	19	13.3	80	Pos
10. HH	F	NK	? colitis	71	19.8	77	Pos
11. JS	M	82	Pneumonia	72	28.9	82	Neg
12. ML	F	69	? intra-abdominal abscess	15	1.5	32	Neg
13. WS	M	49	? intra-abdominal abscess	54	30.6	85	Neg
14. AG	F	80	Pneumonia	54	39.6	73	Pos
15. JG	M	65	? infected Y-graft	43	10.7	41	Neg
16. AC	F	59	? occult infection	82	36.6	69	Neg
17. JB	F	29	? intra-abdominal abscess	56	52.3	58	Neg

TABLE 3(i) (cont'd)

PATIENT DETAILS, NEUTROPHIL RECOVERY, LABELLING EFFICIENCY AND SCAN OUTCOME

PATIENT	SEX	AGE	REASON FOR SCAN	NEUTROPHIL RECOVERY (%)	NEUTROPHIL RECOVERY ($x10^7$)	LABELLING (%)	SCAN Pos/Neg
18. HC	M	67	? intra-abdominal collection	59	24.6	57	Neg
19. ML	F	63	? mycotic aneurysm	27	12.7	68	Pos
20. WB	M	37	Pneumonia	49	49.0	89	Neg
21. DO	M	NK	? occult infection	60	11.6	51	Neg
22. RL	M	66	? occult infection	53	15.4	82	Neg
23. AK	M	78	? intra-abdominal sepsis	91	23.8	71	Neg
24. DP	M	22	? intra-abdominal sepsis (kinetic study)	70	8.7	51	Neg
25. WC	M	59	? infected Y-graft (kinetic study)	43	15.1	66	Neg
26. CI	M	54	Kinetic study	44	13.8	63	Neg
27. CD	M	50	? wound abscess	64	14.5	63	Neg
28. ML	F	39	Post-operative pyrexia	36	31.0	75	Neg
29. ML	F	55	? thrombophlebitis	56	21.2	85	Neg
30. DB	M	30	? myocarditis	71	7.5	63	Neg
31. WD	F	68	Myocardial infarct	23	13.2	81	Neg
32. WS	M	64	Myocardial infarct	38	31.9	81	Pos
33. IL	M	61	Myocardial infarct	47	26.6	80	Pos
34. EF	F	78	Myocardial infarct	58	27.9	76	Pos

TABLE 3(i) (cont'd)

PATIENT DETAILS, NEUTROPHIL RECOVERY, LABELLING EFFICIENCY AND SCAN OUTCOME

PATIENT	SEX	AGE	REASON FOR SCAN	NEUTROPHIL RECOVERY (%)	NEUTROPHIL RECOVERY ($x10^7$)	LABELLING (%)	SCAN Pos/Neg
35. WR	M	56	Myocardial infarct	46	23.5	65	Pos
36. AS	M	58	Myocardial infarct	32	17.1	77	Pos
37. RH	M	52	Myocardial infarct	33	15.5	78	Neg
38. GB	M	68	Myocardial infarct	45	36.1	81	Pos
39. PM	M	65	Myocardial infarct	55	18.6	75	Pos
40. DW	M	61	Myocardial infarct	56	21.8	70	Neg
41. WM	M	64	Myocardial infarct	42	20.7	73	Pos
42. WI	F	N/A	Myocardial infarct	60	29.4	89	Neg
43. AA	M	64	Kinetic study	65	18.1	72	Neg
44. CK	M	68	Kinetic study	48	19.8	70	Neg
45. AD	M	82	Kinetic study	40	25.7	74	Neg
46. HG	F	62	Infective exacerbation of chronic bronchitis			FAIL	
47. AD	M	57	Pyrexial ? cause Chronic bronchitis			FAIL	
48. GS	M	65	Chronic bronchitis			FAIL	
49. HL	F	77	? occult infection			FAIL	
50. EB	F	85	? abdominal sepsis			FAIL	

TABLE 3 (ii) KINETIC DATA

INDIUM COUNTS FOR EACH ORGAN CORRECTED FOR TIME EXPRESSED AS A PERCENTAGE OF MAXIMUM WHOLE FIELD OF VIEW COUNTS FOR THE 8 SUBJECTS AT PROGRESSIVELY INCREASING TIME INTERVALS

HEART

TIME (secs)	S	B	F	Mc	H	M	D	I
2	0.2	0.3	1.2	0.7	0.7	0.4	1.6	11.8
7	5.3	18.0	12.6	7.3	3.1	5.3	11.9	5.0
12	4.7	8.7	8.0	5.0	3.3	5.6	9.8	5.2
17	4.1	6.1	8.6	3.0	3.3	5.8	7.2	3.4
22	2.9	4.5	4.5	2.0	3.0	3.7	5.3	3.6
27	1.7	15.0	3.5	1.7	2.0	2.7	5.0	3.0
35	1.4	4.1	3.7	1.9	1.8	2.2	4.4	2.8
45	1.8	3.8	4.7	2.1	2.2	2.3	4.6	2.9
55	1.8	3.7	4.7	2.4	2.4	2.2	4.4	2.9
65	1.7	3.1	4.7	2.3	2.4	2.4	4.4	2.8
75	1.5	3.4	4.6	2.3	2.2	2.4	4.0	2.7
85	1.5	3.3	4.1	2.2	1.9	2.0	4.0	2.3
95	1.3	3.6	4.2	2.3	1.6	1.9	3.9	2.6
105	1.3	3.0	4.0	2.2	1.9	1.8	4.3	2.2
115	1.4	2.9	3.7	2.0	1.8	1.8	3.9	2.4
130	1.3	3.0	3.6	2.3	1.6	1.8	3.8	2.1
150	1.3	2.9	3.5	2.3	1.5	1.7	3.5	2.1
170	1.2	2.8	3.4	2.0	1.6	1.6	3.5	2.0
190	1.1	2.6	3.3	2.0	1.6	1.5	3.3	2.1
210	1.1	2.6	3.3	2.1	1.6	1.6	3.4	2.2
230	1.0	2.6	3.3	2.2	1.6	1.4	3.2	2.0

TABLE 3 (ii) cont'd

HEART

TIME (secs)	S	B	F	Mc	H	M	D	I
255	1.0	2.6	3.2	2.0	1.4	1.4	3.1	2.1
285	1.0	2.6	3.0	1.9	1.5	1.4	3.1	2.0
315	1.1	2.6	3.1	1.9	1.5	1.4	3.1	2.0
345	1.1	2.5	3.1	2.0	1.5	1.3	2.8	1.9
375	1.1	2.5	2.9	1.8	1.6	1.3	3.0	2.0
405	1.0	2.4	2.9	1.7	1.5	1.2	2.9	1.9
435	1.0	2.4	2.9	1.8	1.5	1.2	2.8	1.8
465	1.0	2.5	2.9	2.1	1.5	1.2	2.9	1.7
495	1.0	2.3	2.9	1.8	1.4	1.2	3.0	1.7
525	1.0	2.4	2.9	1.9	1.5	1.3	2.9	1.8
555	1.0	2.3	2.8	1.7	1.4	1.2	2.9	1.7
585	1.0	2.3	2.8	1.8	1.4	1.2	2.8	1.8
630	0.9	2.2	2.9	1.8	1.5	1.2	2.7	1.8
690	1.0	2.3	2.9	1.8	1.4	1.2	3.0	1.8
750	0.9	2.3	2.9	1.7	1.4	1.1	2.7	1.7
810	0.9	2.2	3.0	1.6	1.5	1.1	2.8	1.7
870	1.0	2.2	3.0	1.7	1.5	1.2	2.8	1.7
930	1.0	2.2	3.0	1.7	1.4	1.2	2.7	1.7
990	1.0	2.1	3.0	1.7	1.4	1.2	2.7	1.6
1050	0.9	2.1	3.0	1.6	1.5	1.2	2.7	1.6
1110	0.9	2.1	3.1	1.6	1.5	1.1	2.7	1.6
1170	0.9	1.9	3.1	1.7	1.5	1.2	2.6	1.6
1230	0.9	2.0	3.1	1.7	1.5	1.2	2.7	1.6
1290	1.0	1.9	3.1	1.6	1.4	1.1	2.8	1.6
1350	0.9	1.9	3.2	1.6	1.4	1.2	2.7	1.7

TABLE 3 (ii) cont'd

HEART

TIME (secs)	S	B	F	Mc	H	M	D	I
1410	0.9	1.8	3.2	1.6	1.4	1.1	2.8	1.6
1470	0.9	1.8	3.2	1.6	1.4	1.1	2.8	1.7
1529	0.9	1.8	3.2	1.7	1.4	1.1	2.8	1.5

TABLE 3 (ii) **KINETIC DATA**

LUNGS

TIME (secs)	S	B	F	Mc	H	M	D	I
2	3.8	0.9	3.9	3.1	2.1	2.1	3.0	10.8
7	23.0	21.6	32.1	35.9	21.1	17.6	21.1	33.1
12	32.3	35.1	45.5	55.9	34.7	32.4	31.5	26.8
17	29.4	26.2	33.9	45.4	51.1	23.8	18.1	21.1
22	23.6	21.1	30.5	41.7	31.9	16.5	16.6	23.6
27	20.5	20.8	31.4	42.1	30.2	16.4	15.5	24.9
35	20.6	20.8	33.8	45.0	27.3	18.5	16.4	25.4
45	20.9	19.9	30.1	43.4	26.5	17.5	14.8	23.3
55	19.4	19.1	25.8	37.2	22.9	16.2	13.4	21.7
65	17.8	19.4	23.2	33.0	19.6	15.6	12.7	20.4
75	18.3	18.6	21.9	30.6	19.3	14.8	13.2	19.7
85	18.4	18.6	21.2	29.8	18.1	14.6	13.0	18.9
95	18.7	18.7	21.4	28.9	17.9	14.5	13.0	18.6
105	17.7	18.6	19.7	28.0	16.7	13.8	12.4	18.7
115	17.8	17.4	20.1	26.1	18.2	13.6	12.4	18.4
130	18.3	18.2	19.5	26.0	17.3	13.5	12.3	18.0
150	18.5	18.6	19.1	25.9	17.2	13.2	12.7	17.7
170	17.8	18.4	18.5	24.1	15.9	13.0	12.4	17.9
190	17.6	18.7	18.5	23.5	16.4	12.5	11.9	18.3
210	17.8	18.9	17.6	22.1	15.3	11.6	12.3	17.8
230	17.2	19.0	17.5	21.1	15.2	11.7	12.0	16.9
255	16.7	19.0	17.0	20.3	14.3	11.4	11.6	17.3
285	16.6	19.0	15.8	20.0	13.9	10.7	11.3	16.7
315	15.7	18.8	15.3	19.2	13.0	10.6	11.3	16.0

TABLE 3 (ii) cont'd

LUNGS

TIME (secs)	S	B	F	Mc	H	M	D	I
345	15.4	18.7	14.5	18.4	12.4	10.2	10.8	15.4
375	14.9	18.3	14.1	17.8	12.0	9.6	11.2	15.1
405	13.9	18.1	13.4	17.4	11.0	9.4	10.6	14.8
435	13.9	17.8	13.0	17.0	10.9	9.2	10.5	14.4
465	13.4	17.9	12.9	16.6	9.8	9.0	10.3	14.2
495	13.0	17.5	12.2	16.1	9.9	8.7	10.3	14.0
525	12.9	17.4	11.8	16.1	9.8	8.6	10.0	13.1
555	12.4	17.3	11.5	16.2	9.5	8.4	10.0	13.4
585	12.2	16.7	11.1	15.4	8.9	8.3	9.8	12.7
630	11.6	15.8	10.9	15.0	9.0	8.0	9.7	13.0
690	11.4	15.4	10.7	14.6	8.3	8.0	9.4	12.5
750	10.9	14.4	10.4	14.2	8.5	7.6	9.4	12.1
810	10.5	13.9	10.2	13.8	8.4	7.6	9.3	11.9
870	10.2	13.1	10.4	13.3	7.8	7.4	9.4	11.4
930	10.0	12.2	9.9	13.1	7.7	7.4	9.2	11.4
990	9.5	11.6	10.0	12.7	7.8	7.1	9.0	11.2
1050	9.5	11.0	10.0	12.4	7.8	6.9	9.0	11.0
1110	9.1	10.6	9.9	12.6	7.7	6.8	8.9	11.0
1170	9.1	10.0	9.7	12.7	7.3	6.8	9.0	10.6
1230	8.8	9.7	9.8	12.8	7.1	6.7	8.6	10.3
1290	8.4	9.4	10.0	12.6	7.3	6.6	8.6	10.4
1350	8.3	9.1	9.6	12.6	6.8	6.5	8.4	10.5
1410	8.1	8.5	9.6	12.5	6.7	6.4	8.3	10.0
1470	8.0	8.2	9.7	12.5	7.0	6.2	8.4	10.1
1529	7.8	8.0	9.6	12.4	6.9	6.1	8.3	9.9

TABLE 3 (ii) **KINETIC DATA**

LIVER

TIME (secs)	S	B	F	Mc	H	M	D	I
2	0.5	0.6	0.2	0.3	0.7	2.4	1.2	1.4
7	2.0	1.4	0.8	0.8	1.7	2.6	1.9	3.1
12	2.1	1.6	0.8	1.2	1.8	3.4	2.2	2.4
17	2.2	2.4	1.1	1.5	1.4	3.4	3.2	3.2
22	2.6	4.1	2.0	1.7	2.0	4.2	5.0	3.7
27	2.6	5.5	2.4	1.7	2.9	5.6	7.6	4.5
35	4.2	6.7	3.1	2.2	3.4	8.6	9.9	5.2
45	5.0	7.8	3.8	2.4	3.8	10.8	10.4	5.8
55	5.9	8.4	4.0	2.2	4.8	11.2	10.9	6.7
65	6.3	9.1	4.7	2.3	5.6	11.9	11.2	7.9
75	7.0	9.6	4.8	2.4	6.4	13.4	12.0	8.1
85	7.1	10.7	5.3	2.4	6.6	14.0	11.7	9.4
95	7.6	11.1	5.8	2.4	6.7	14.0	12.3	9.8
105	7.9	11.6	5.8	2.7	8.4	14.9	13.0	9.8
115	8.4	11.6	6.0	2.5	8.0	15.7	13.8	10.1
130	8.5	12.6	6.4	2.7	8.2	15.4	14.3	10.4
150	9.1	13.0	6.9	2.7	8.8	17.9	15.2	10.9
170	9.6	13.8	7.1	2.8	9.5	18.7	15.7	11.4
190	9.8	14.1	7.4	2.8	10.1	19.7	16.4	11.4
210	10.0	14.5	7.8	2.8	10.8	20.6	16.7	11.8
230	10.3	14.5	8.2	2.9	11.6	21.4	17.2	12.5
255	10.4	15.2	8.2	3.0	11.6	22.2	17.7	13.2
285	10.8	15.5	8.7	2.8	12.3	23.1	18.2	13.8
315	11.0	16.2	8.6	2.9	13.1	23.8	18.2	14.3

TABLE 3 (ii) cont'd

LIVER

TIME (secs)	S	B	F	Mc	H	M	D	I
345	11.2	16.2	9.1	2.7	12.9	24.5	18.9	15.0
375	11.6	16.3	9.2	2.7	13.7	25.1	18.9	15.1
405	11.6	16.3	9.4	2.8	13.8	25.4	19.1	16.2
435	12.0	16.8	9.5	2.8	14.4	25.5	19.4	16.5
465	11.9	17.0	9.6	2.9	14.4	25.7	19.9	16.9
495	12.3	17.0	9.7	2.7	15.0	25.8	19.6	16.9
525	12.2	17.4	10.0	2.8	14.9	26.2	20.1	17.5
555	12.1	17.4	9.9	2.7	14.9	26.5	19.9	18.3
585	12.2	17.6	10.1	2.7	15.0	26.7	19.5	18.3
630	12.1	18.1	9.8	2.8	15.8	26.6	20.0	18.7
690	12.2	18.9	9.8	2.7	16.0	26.9	20.1	19.2
750	12.4	18.9	9.6	2.7	15.7	27.2	19.8	19.4
810	12.3	19.5	9.6	2.7	16.0	27.2	19.8	19.6
870	12.2	19.7	9.5	2.7	16.0	27.1	19.4	20.0
930	12.4	20.0	9.4	2.7	16.0	27.3	19.5	20.0
990	12.1	20.7	9.3	2.7	15.8	27.2	19.1	20.3
1050	12.2	21.0	9.1	2.7	16.0	27.2	18.8	20.5
1110	12.0	21.1	9.0	2.7	15.8	27.2	19.0	21.3
1170	12.1	21.4	8.8	2.7	16.3	26.9	18.9	21.4
1230	12.2	22.0	8.6	2.8	15.8	26.8	18.6	21.8
1290	12.0	22.0	8.5	2.7	16.0	26.8	18.8	21.8
1350	12.1	22.5	8.4	2.7	15.3	26.8	18.6	21.8
1410	12.1	22.8	8.5	2.7	15.3	26.7	18.4	21.9
1470	12.1	22.9	8.2	2.7	15.2	26.8	18.2	21.9
1529	12.1	23.4	8.1	2.7	15.2	26.8	18.2	22.3

TABLE 3 (ii) **KINETIC DATA**

SPLEEN

TIME (secs)	S	B	F	Mc	H	M	D	I
2	0.0	0.1	0.1	0.2	0.1	0.0	0.1	0.0
7	0.1	0.2	0.3	0.9	0.2	0.3	0.9	0.7
17	0.6	0.8	1.1	3.1	0.8	1.5	2.9	1.5
22	0.7	1.1	1.5	3.3	0.8	1.5	3.2	1.6
27	1.0	1.1	1.5	3.3	1.0	1.8	2.9	1.6
35	1.0	1.3	1.6	3.1	0.9	1.5	3.0	1.8
45	1.0	1.4	1.6	3.8	0.8	1.5	3.0	1.9
55	0.9	1.5	1.8	4.4	0.8	1.7	3.3	2.1
65	1.2	1.8	2.0	4.6	1.0	1.6	3.4	2.3
75	1.2	1.6	2.1	4.8	0.9	1.8	3.5	2.3
85	1.4	2.0	2.2	5.0	1.0	1.8	3.5	2.4
95	1.4	2.2	2.5	5.3	1.0	2.0	3.4	2.2
105	1.3	2.2	2.5	5.5	1.1	2.1	3.6	2.6
115	1.2	2.3	2.6	6.0	1.2	2.1	4.1	2.9
130	1.4	2.5	2.9	6.5	1.2	2.3	3.9	3.0
150	1.5	2.4	3.1	6.8	1.2	2.4	4.0	3.4
170	1.6	2.7	3.4	7.2	1.3	2.4	4.1	3.3
190	1.6	2.8	3.6	7.7	1.0	2.7	4.5	3.5
210	1.6	2.7	3.7	8.5	1.4	2.6	4.6	3.5
230	1.8	2.8	3.9	8.7	1.1	2.7	4.6	3.5
255	2.0	2.9	4.0	8.9	1.4	2.8	5.0	3.9
285	2.0	3.0	4.3	9.4	1.3	2.8	5.3	3.9
315	2.1	3.1	4.6	9.8	1.3	3.0	5.5	4.0
345	2.2	3.1	4.8	10.2	1.3	3.1	5.6	4.3

TABLE 3 (ii) cont'd

SPLEEN

TIME (secs)	S	B	F	Mc	H	M	D	I
375	2.3	3.1	5.1	10.4	1.5	3.1	5.7	4.4
405	2.4	3.3	5.2	10.6	1.4	3.4	5.6	4.6
435	2.6	3.5	5.4	10.8	1.5	3.4	5.8	4.6
465	2.6	3.5	5.4	11.0	1.6	3.6	5.9	4.9
495	2.7	3.6	5.5	11.0	1.5	3.6	6.1	4.7
525	2.8	3.5	5.7	11.3	1.6	3.7	6.1	5.0
555	2.8	3.8	5.8	11.3	1.7	3.6	6.2	5.0
585	3.0	3.6	5.9	11.2	1.8	3.8	6.4	5.1
630	3.1	3.7	6.1	11.5	1.6	3.9	6.5	5.2
690	3.4	3.9	6.3	11.9	1.9	4.0	6.8	5.2
750	3.4	4.0	6.3	11.9	1.8	4.0	6.9	5.3
810	3.6	4.0	6.7	12.3	1.8	4.3	7.1	5.4
870	3.8	4.3	6.8	12.5	2.0	4.3	7.3	5.4
930	3.9	4.5	6.9	12.6	1.9	4.5	7.2	5.5
990	4.1	4.5	7.1	12.6	2.1	4.5	7.5	5.7
1050	4.1	4.5	7.2	12.5	2.2	4.5	7.8	5.5
1110	4.2	4.6	7.2	12.6	2.0	4.7	7.9	5.4
1170	4.4	4.7	7.3	12.6	2.1	4.8	7.8	5.4
1230	4.6	4.8	7.5	12.7	2.1	4.8	8.0	5.5
1290	4.6	4.8	7.4	12.7	2.1	4.9	7.9	5.5
1350	4.8	5.1	7.6	12.7	2.2	5.0	8.2	5.6
1410	4.9	5.2	7.7	12.6	2.3	5.2	8.3	5.5
1470	5.1	5.3	7.8	12.6	2.3	5.1	8.3	5.5
1529	5.1	5.3	7.8	12.5	2.3	5.1	8.6	5.4

TABLE 4 (i)

PATIENT DETAILS AND RESULTS OF IMAGING WITH INDIUM-111 LABELLED AUTOLOGOUS NEUTROPHILS

	Positive image (n = 23)	Negative image (n = 7)	Statistical significance
Age (years)	62.0 (10.8)	59.8 (9.4)	NS (1)
Sex	(6F; 17M)	(2F; 5M)	NS (2)
Location of acute myocardial infarct	10 inf; 13 ant	3 inf; 4 ant	NS (2)
Interval from onset of chest pain to injection of ^{111}In neutrophils (h)	20.3 (6.4)	27.6 (5.8)	$p < 0.02$ (3)
Peak creatine kinase (u/l)	2023.5 (916.0)	1825 (1214.0)	NS (1)
Total leucocyte count (10^9/l)	12.9 (3.2)	12.5 (3.4)	NS (1)
Number of neutrophils injected (10^8/l)	2.7 (0.9)	2.6 (0.7)	NS (1)
Activity of ^{111}In administered (MBq)	32.8 (8.4)	29.4 (8.2)	NS (1)
Drugs administered:			
Non-steroidal anti-inflammatory drugs	5	1	
Lignocaine	2	3	
Calcium antagonists	1	2	
Nitrates	7	1	

All values are shown as mean (SD)

(1) Unpaired t test

(2) Exact probability test

(3) Unpaired Wilcoxon rank sum test

TABLE 5 (i)

PATIENT DETAILS AND RESULTS SOF ^{111}In LABELLED NEUTROPHIL AND ^{99M}Tc-PYROPHOSPHATE IMAGING IN PATIENTS WITH ACUTE MYOCARDIAL INFARCTION TREATED WITHOUT THROMBOLYSIS

Patient No	Age (yrs)	^{111}In (voxels)	^{99m}Tc-PYP (voxels)	Ratio	LVEF (%)	CK (u/litre)	Time to infection (hrs)	Lig (Y/N)	Steroid (Y/N)
1	61	276	129	2.14	dec'd	2477	8	N	N
2	57	85	114	0.75	34	1995	7.5	N	N
3	41	144	188	0.77	25	6255	6.5	N	N
4	57	19	319	0.06	25	2950	10.0	Y	N
5	54	29	33	0.88	71	522	6.5	N	N
6	58	133	173	0.77	32	1532	9.5	N	N
7	68	69	77	0.90	51	1911	14.5	Y	N
8	64	187	169	1.11	40	1638	7.0	N	N
9	63	74	405	0.18	15	3715	8.5	Y	N
10	64	131	399	0.33	27	2091	4.5	N	N

TABLE 5 (ii)

PATIENT DETAILS AND RESULTS OF ^{111}In-LABELLED NEUTROPHIL AND ^{99m}Tc-PYROPHOSPHATE IMAGING IN PATIENTS WITH ACUTE MYOCARDIAL INFARCTION TREATED WITH THROMBOLYSIS

Patient No	Age (yrs)	^{111}In (voxels)	^{99m}Tc-PYP (voxels)	Ratio	LVEF (%)	CK (u/litre)	Time to infection (hrs)		Lig (Y/N)	Steroid (Y/N)
11	30	78	323	0.24	40	4225	12	APSAC	N	N
12	63	106	111	0.96	52	1419	5	STREP	N	Y
13	65	134	229	0.58	35	1625	18	STREP	N	Y
14	44	0	190	0	56	533	7.5	APSAC	Y	N
15	38	75	192	0.39	29	6955	15	APSAC	Y	N
16	63	0	153	0	42	1364	14	APSAC	N	N
17	47	98	272	0.36	27	2059	9	STREP	Y	Y
18	67	74	111	0.67	68	1516	10.5	STREP	N	Y
19	53	153	268	0.57	24	1719	5.5	STREP	N	Y
20	62	13	297	0.04	25	4020	14	APSAC	N	N
21	55	160	238	0.67	26	2073	6	APSAC	N	N

TABLE 5(iii)

SUMMARY OF DETAILS OF PATIENTS TREATED WITH AND WITHOUT THROMBOLYSIS WHO WERE IMAGED WITH BOTH ^{111}IN-NEUTROPHILS AND ^{99m}TC PYROPHOSPHATE

	CK max u/litre	LVEF (%)	Time to injection	^{111}In (voxels)	^{99m}Tc (voxels)	^{111}In/^{99m}Tc
No thrombolysis (n = 10)	2508 (522-6255)	36 (15-51)	8 (4.5-14.5)	114 (19-276)	201 (77-405)	0.79 (0.06-2.14)
Thrombolysis (n = 11)	2500 (533-6955)	40 (27-68)	11 (5-18)	81 (0-160)	217 (111-323)	0.41 (0-0.96)
	NS	NS	NS	NS	NS	$p < 0.05$

TABLE 6 (i)

CHARACTERSTICS OF NORMAL SUBJECTS, PATIENTS WITH CHRONIC ISCHAEMIC HEART DISEASE AND PATIENTS WITH MYOCARDIAL INFARCTION

			MYOCARDIAL INFARCTION	
	NORMALS n = 35	IHD n = 30	NO THROMBOLYSIS n = 15	THROMBOLYSIS n = 17
Age	34 (22-63)	59 (37-76)	58 (38-74)	56 (30-69)
Male/female	30:5	24:6	10:5	13:4
WBC (x 10^9/l)	5.8 (3.4-9.3)	6.7 (5.2-12.6)	13.2 (9.6-23.0)	16.8 (7.9-33.7)
LVEF (%)	-	41 (18-63)	32 (15-71)	40 (24-68)
Peak creatine kinase	-	-	1635 (522-6255)	2059 (533-6955)
Streptokinase/APSAC	-	-	-	9/8
Lignocaine	-	-	3	5
Hydrocortisone	-	-	-	9
Deaths	-	-	2	1

TABLE 6(ii)

STATISTICAL RESULTS FOR NEUTROPHIL ELASTASE COMPARING THROMBOLYTIC AND NO-THROMBOLYTIC THERAPY OVER TIME WITH CONTROL SUBJECTS

	8 hours	16 hours	24 hours	32 hours	40 hours	48 hours
No thrombolysis	$p < 0.0006$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0005$
Thrombolysis	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0003$

TABLE 6(iii)

STATISTICAL RESULTS FOR NEUTROPHIL ELASTASE COMPARING THROMBOLYTIC AND NO-THROMBOLYTIC THERAPY OVER TIME WITH PATIENTS WITH ISCHAEMIC HEART DISEASE

	8 hours	16 hours	24 hours	32 hours	40 hours	48 hours
No thrombolysis	NS	$p < 0.012$	$p < 0.05$	$p < 0.015$	$p < 0.0001$	$p < 0.043$
Thrombolysis	$p < 0.0001$	$p < 0.0002$	$p < 0.002$	$p < 0.0001$	$p < 0.041$	NS

TABLE 7 (i)

ENDOGENOUS ANTIOXIDANT DEFENCE MECHANISMS

ANTIOXIDANT	MECHANISM	EFFECT ON LIPID PEROXIDATION
Vitamin C	Scavenges free radicals	Yes
Vitamin E	Scavenges free radicals	No
Glutathione peroxidase	Catalyses H_2O_2 to H_2O	Yes
Catalase	Catalyses H_2O_2 to H_2O	No
Superoxide dismutase	Catalyses O_2^- to H_2O_2	No
Lactoferrin/ transferrin	Bind iron salts	Yes
Caeruloplasmin	Mechanism not understood	Yes

TABLE 7 (ii)

STATISTICAL RESULTS FOR PL-9, 11-LA' COMPARING THROMBOLYTIC AND NO-THROMBOLYTIC THERAPY OVER TIME WITH CONTROL SUBJECTS

	8 hours	16 hours	24 hours	32 hours	40 hours	48 hours
No thrombolysis	$p < 0.0019$	$p < 0.011$	$p < 0.003$	$p < 0.0057$	$p < 0.01$	$p < 0.017$
Thrombolysis	$p < 0.001$	$p < 0.0001$	$p < 0.0014$	$p < 0.0006$	$p < 0.002$	$p < 0.007$

TABLE 7 (iii)

STATISTICAL RESULTS FOR PL-9, 11-LA' COMPARING THROMBOLYTIC AND NO-THROMBOLYTIC THERAPY OVER TIME WITH PATIENTS WITH ISCHAEMIC HEART DISEASE

	8 hours	16 hours	24 hours	32 hours	40 hours	48 hours
No thrombolysis	$p < 0.02$	$p < 0.038$	$p < 0.023$	$p < 0.05$	NS	NS
Thrombolysis	$p < 0.0084$	$p < 0.0017$	$p < 0.023$	$p < 0.013$	$p < 0.045$	NS

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The preparation and *in vivo* behaviour of ^{111}In-oxine labelled neutrophils separated from whole blood using mono-poly resolving medium

D. BELL[1], A.M. MILLAR[2], M. McGILLIVRAY[1] and A.L. MUIR[1]

University Department of [1]Medicine and [2]Radiopharmacy, Royal Infirmary, Edinburgh, UK

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Summary

Using a single step separation procedure, we have developed a method for labelling human neutrophils with ^{111}In-oxine. This method allows a rapid separation of neutrophils from whole blood, with negligible mononuclear or red cell contamination. Preliminary studies using ^{111}In-labelled neutrophils show minimal lung retention and early accumulation in the spleen consistent with viable cells. In addition, focal accumulation of ^{111}In has been imaged in patients with localized inflammation or sepsis.

Introduction

Indium-111 (^{111}In) labelled leucocyte imaging has become a common investigation for the localization of inflammatory lesions [1–5]. The neutrophils are the predominant cell involved in the inflammatory response and it has therefore been suggested that a pure preparation of neutrophils is superior to mixed leucocytes for *in vivo* studies using ^{111}In-labelled cells [6]. The use of pure neutrophils also has the advantage of avoiding the labelling of the radiosensitive lymphocytes [7–9].

A number of methods exist to isolate pure neutrophils from blood but at present these are time consuming and involve several steps [10, 11]. Mono-poly Resolving Medium is a commercially available medium which can be used to isolate neutrophils from whole blood in a single step. In this paper we describe the isolation of neutrophils by this technique and their subsequent labelling with ^{111}In-oxine. Kinetic data and positive clinical studies are also presented.

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Methods

Patients

Investigations with ^{111}In-neutrophils were performed on 45 patients with a variety of suspected inflammatory clinical conditions. Neutrophil kinetics were studied in eight of the patients with no evidence of active lung disease.

Cell separation

Venous blood (60 ml) was collected aseptically via a 19G infusion set into a syringe containing 300 units of preservative-free heparin. A full blood count and ESR were performed on 10 ml blood. All subsequent procedures were performed using aseptic technique. In duplicate, 25 ml blood was layered over 12 ml Mono-poly Resolving Medium (Flow Laboratories) in a sterile tube and centrifuged at 400 g for 60 min. Differential migration during centrifugation results in two distinct cell bands and a red cell pellet (Fig. 1). From the top plasma layer, 8 ml was collected and centrifuged at 1000 g for 10 min to provide platelet-poor plasma (PPP). The remaining plasma and upper cell band containing mononuclear cells were discarded. The neutrophils were recovered from the lower cell band, resuspended in 40 ml phosphate buffered saline pH 7.4 (PBS) and centrifuged at 400 g for 10 min. The supernatant was discarded and the cell pellet was resuspended in 10 ml PBS. A 1 ml sample was taken to determine total and differential white cell counts and assess red cell contamination.

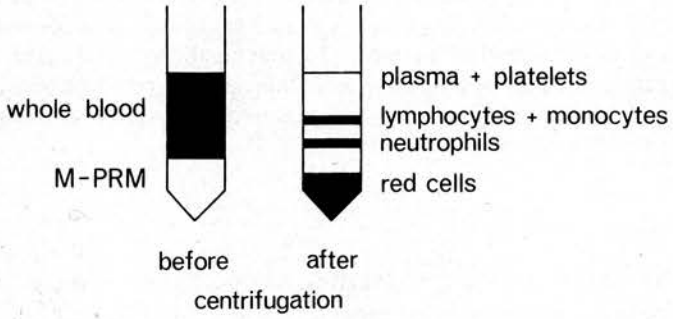


Fig. 1. Diagnostic illustration of blood cell separation on Mono-poly Resolving Medium.

Labelling procedure

^{111}In-oxine solution (20–40 MBq) (Amersham International plc) was added dropwise to the remaining suspension of neutrophils. After incubation at room temperature for 15 min, 3 ml of PPP was added then the cell suspension was centrifuged at 250 g for 10 min. The supernatant was transferred to a fresh tube. The cell pellet was resuspended to a volume of 5 ml with equal parts of PBS and PPP. The activities of the cell suspension and supernatant were measured in a radioisotope calibrator and the labelling efficiency calculated. The cell suspension which contained 18 to 40 MBq ^{111}In and 2.0 to 13.0×10^{7} cells ml^{-1} was drawn into a syringe ready for injection.

Cell counting

All cell counts were performed manually using a new improved Neubauer chamber (0.100 mm). Blood films made from whole blood and the neutrophil suspension were stained with May-Grunwald/Giemsa and a differential cell count determined. The percentage cell recovery was calculated by comparing the neutrophil count in whole blood with that in the neutrophil suspension.

Scintigraphic investigations

Imaging was performed using a large field of view gamma camera (GEC-400T Maxicamera) interfaced to a PDP11-34 computer (Digital Equipment Corporation). Kinetic data were obtained from eight patients in the following manner: the patient was positioned anteriorly to visualize lungs, liver, spleen and heart and the ^{111}In-neutrophil suspension was administered intravenously by a fast running 5% dextrose infusion. A sequence of 64×64 matrix images was taken over 25 min at a varying frame interval starting at 5 s for the first six images and then increasing stepwise to 60 s for the later images. The counts were normalized for frame length. To assess ^{111}In-neutrophil kinetics, the movie images were inspected and regions of interest (ROI) created around the heart, lungs, liver, spleen and whole field of view. The whole field frame showing the maximum count-rate was assumed to represent the total activity injected. The count-rates from the other ROIs were expressed as a percentage of the maximum whole field count-rate and a time–activity curve was created for each organ. This method of data analysis allows direct comparison of the relative activity in each organ. In all patients, static images were obtained at 6 and 24 h after injection.

Results

From the 45 patients' blood samples on which separation was performed, the neutrophil recovery was 47.1% (S.D.±17.5%) and in only one sample was the

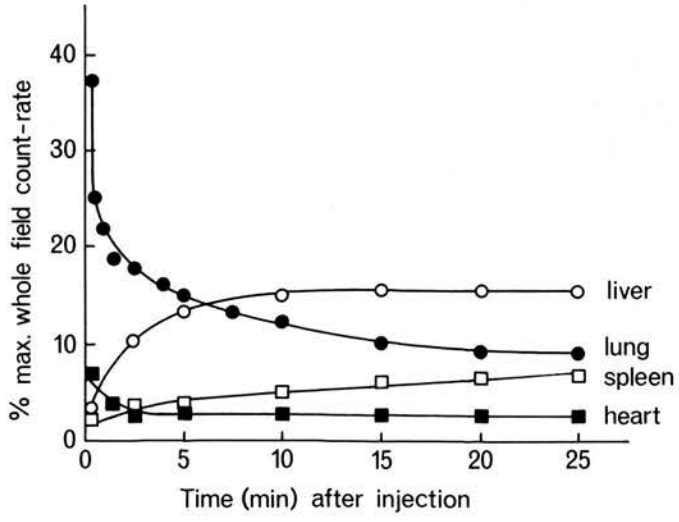
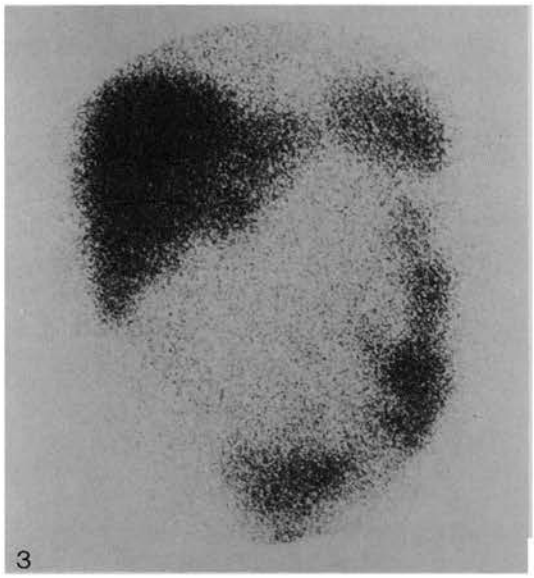
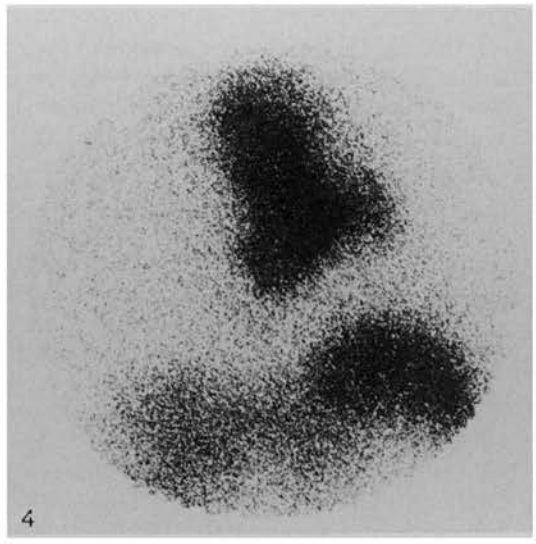


Fig. 2. ^{111}In-neutrophil time–activity curves for lungs, heart, liver and spleen.





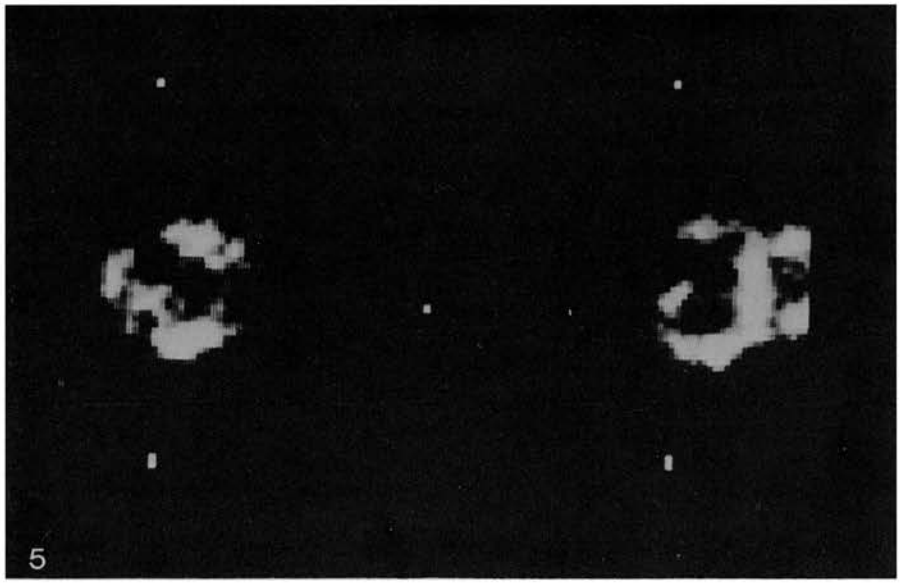


Fig. 3. Gamma camera image (24 h) showing the anterior abdominal view obtained from a patient with ischaemic colitis. Predominant uptake of ^{111}In can be seen in the sigmoid and descending colon.

Fig. 4. Anterior chest image (24 h) showing marked uptake of ^{111}In in the left upper lobe of a patient with obstructive pneumonia in the left upper lobe.

Fig. 5. Reconstructed ^{111}In transverse and coronal head images at 24 h obtained using single photon emission computerized tomography in a patient with a right frontal cerebral abscess.

neutrophil count less than 4.2×10^7. All neutrophil suspensions were labelled with ^{111}In-oxine and a mean labelling efficiency of 72.1% (S.D.±12.9%) was achieved. In addition, red cell contamination was calculated in the eight patients used for kinetic data and was found to be 5.7% (S.D.±2.6). Lymphocyte contamination was found to be less than 0.5%.

Fig. 2 shows the mean ^{111}In-neutrophil kinetic data obtained from the eight patients studied. The data are expressed as percentages of the maximum count rate achieved in the whole field of view. Heart count-rate falls to 3% of the maximum whole field count-rate within 90 s and after 5 min remains constant at 2% for the remainder of the study. Similarly, lung count-rate falls to 19% within the first 90 s and thereafter falls at a slower rate to 9% by 20 min indicating a small degree of lung retention. The liver count-rate rises to 16% by 15 min and remains constant, whereas the spleen count-rate continues to rise throughout the study.

Of the 45 studies, 18 showed positive uptake, three examples of which are given in Figs. 3 to 5. No patient with a negative scan was subsequently demonstrated to have a major site of infection.

Discussion

Leucocytes labelled with a variety of radionuclides have been used to locate infection or inflammation since the early 1970s [12–14]. In recent years, ^{111}In-chelates have been used to label leucocytes for routine diagnostic purposes. While much of the initial work has involved the labelling of mixed leucocytes with ^{111}In, more recently emphasis has been placed on two advantages of using pure neutrophils: (1) avoidance of labelling radiosensitive lymphocytes [7–9]. (2) Reduction in the labelling of non-specific cells principally erythrocytes and platelets [6].

A number of techniques have been developed to isolate and label pure neutrophils with ^{111}In. These methods are time consuming, requiring the preparation of a discontinuous density gradient for the cell separation. In an attempt to retain cell function it has been recommended that the neutrophils be isolated and labelled in a plasma environment. To achieve this, a technique using a discontinuous density gradient prepared with the patient's own plasma and subsequent labelling in plasma with ^{111}In-tropolonate has been developed [10]. Isolation and labelling in this manner does, however, prolong the procedure as the gradient can only be prepared after cell-free plasma has been obtained.

The purpose of this study was not to evaluate the efficacy of ^{111}In-labelled neutrophils as a diagnostic tool as this has been well documented, but to describe a technique for isolating neutrophils directly from whole blood without the need to perform an initial sedimentation step using dextran, hydroxyethyl starch or methyl cellulose as is the current practice [7, 10, 11]. Subsequent labelling of the separated pure neutrophils is achieved with ^{111}In-oxinate. This method does not require the

preparation of a discontinuous density gradient but uses the commercially available Mono-poly Resolving Medium. Good neutrophil recovery is achieved with little red cell and virtually no lymphocyte contamination. Labelling efficiency is excellent and agrees well with other published data for ^{111}In-oxinate [2, 11, 15, 16].

A number of studies has shown that poorly functioning or damaged ^{111}In-labelled neutrophils demonstrate lung retention or liver sequestration [2, 17–20]. It is essential that ^{111}In-labelled cells prepared by any new techniques of cell isolation be assessed for lung retention. One method of achieving this is to compare the passage through the lungs of a simultaneously injected ^{99}Tc^{m}-red blood cells and ^{111}In-neutrophils with simultaneous acquisition in the two energy windows [15]. Using this technique the ^{99}Tc^{m} activity–time curve represents blood flow through the lungs. Retention of ^{111}In-neutrophils is therefore demonstrated by the difference in the lung time–activity curves for the two radionuclides. We decided to use a simpler technique, comparing the ^{111}In activity–time curves for the lungs and heart, the difference in the slope of the two curves demonstrating lung retention. Kinetic data obtained from eight patients in this manner demonstrated early uptake in the liver and spleen, with splenic activity continuing to rise throughout the period of study consistent with functioning cells. Lung clearance of the ^{111}In-labelled neutrophils occurs rapidly, 80% clearing within 90 s. When the lung and heart clearance curves are compared, retention of ^{111}In in the lungs is seen, consistent with the margination of neutrophils within the pulmonary vasculature. Evidence of retention is minimal by 20 min. The difference in total lung and heart counts at 25 min can largely be explained on the basis of differences in pulmonary and cardiac blood volume and need not represent continuing lung retention of ^{111}In-labelled neutrophils. This corresponds well with the kinetic data reported in previous studies [15, 20], showing little lung hold-up with rapid pooling of neutrophils within the spleen. Further confirmation that the cells are functionally viable is provided by the positive scans obtained from a group of patients with a variety of infective and inflammatory conditions, examples of which are given earlier.

In conclusion, we have demonstrated a rapid single-step separation procedure for the isolation of pure neutrophils from whole blood using Mono-poly Resolving Medium. This method is rapid and involves less handling of cells, thus reducing the risk of mechanical damage and requiring less technical expertise. Pure neutrophils separated by this technique have good *in vivo* kinetics and appear functionally normal.

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The acute inflammatory response to myocardial infarction: imaging with indium-111 labelled autologous neutrophils

D BELL, M JACKSON, A M MILLAR, J J NICOLL, M CONNELL, A L MUIR

British Medical Association, Tavistock Square, London WC1H 9JR

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ELL,* M JACKSON,* A M MILLAR,‡ J J NICOLL,† M CONNELL,† MUIR*

*the *Departments of Medicine, †Medical Physics, and ‡Radiopharmacy, Royal Infirmary, Edinburgh*

MARY The uptake of indium-111 labelled neutrophils was examined in 30 patients with e myocardial infarction by planar imaging and single photon emission computed tomohy. The time from venepuncture to reinjection of the autologous labelled neutrophils was 5 hours and imaging was carried out 24 hours later. Twenty three patients had a positive ke of neutrophils in the myocardium and imaging was improved by single photon emission puted tomography. There was a significant difference between the intervals from the onset of pain to injection of labelled neutrophils between patients with positive and negative images; reinjection was more likely to produce a positive image. Indeed, all nine patients reinjected in 18 hours of the onset of symptoms had positive images.

ne results suggest that the stimulus for activation and migration of neutrophils is transient; s an important factor if neutrophil release products play a role in cell damage after coronary sion.

myocardial infarction, myocardial cell death damage produce an acute inflammatory nse characterised by the migration of neutrointo the area of infarcted muscle. Histological ination shows neutrophil infiltration into the ted area within 24 hours and the response is nal at 4–5 days.[1 2] As part of the inflammatory nse, neutrophils release oxygen derived free ls and proteolytic enzymes that in certain circtances may increase tissue injury. To date, the ophil has been implicated in damage to pulry capillaries in adult respiratory distress syne and may also contribute to the pathogenesis physema. No definite role has been established e neutrophil extending myocardial damage in but in animal models of myocardial infarction, t size can be limited by neutrophil tion.[3 4]

hough experimental studies have shown e of indium-111 (^{111}In) labelled neutrophils in ted myocardium[5 6] studies in man have pro conflicting results. McDougall *et al* did not detect uptake of labelled cells in three patients with acute infarction.[7] The time of injection of labelled cells may be of importance, however, because Davies and colleagues obtained positive images when the time to reinjection was short.[8] They also found that positive images were more likely in younger patients.

We have used ^{111}In labelled autologous neutrophils in 24 patients with acute myocardial infarction. Our results confirm the importance of early injection of the neutrophils in obtaining a positive image. We also found that reconstructional imaging from single emission photon computed tomography can be used to resolve difficulties in the interpretation of planar imaging.

sts for reprints to Dr D Bell, Department of Medicine, Royal ary, Edinburgh EH3 9YW.

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Patients and methods

PATIENTS

We studied 30 patients with a diagnosis of acute myocardial infarction based on a history of prolonged ischaemic chest pain (> 30 minutes), electrocardiographic changes associated with myocardial infarction, and a rise in serum creatine kinase to at least twice the upper limit of normal. All gave informed consent, and the study had the approval of

our institute's ethical committee. Table 1 shows patient details and a full record of the drugs administered within the first 24 hours of myocardial infarction.

PREPARATION OF ^{111}IN LABELLED NEUTROPHILS

Autologous neutrophils were separated from whole venous blood and labelled with ^{111}In-oxine.[9] We used an aseptic technique to withdraw whole blood (60 ml) into a syringe containing 300 units of preservative free heparin. Duplicate samples (25 ml) of blood were layered over 12 ml of mono-poly resolving medium (Flow laboratories) in a sterile tube and centrifuged at 400*g* for 60 minutes. This produced a top plasma layer, two distinct cell bands, and a red cell pellet. We collected 8 ml from the top plasma layer and centrifuged it at 1000*g* for 10 minutes to obtain platelet poor plasma. The remaining plasma and upper cell band were discarded and the neutrophils were recovered from the lower cell band. The neutrophils were washed by diluting the recovered cell suspension to 40 ml with phosphate buffered saline pH 7·4, centrifuging at 400*g* for 10 minutes, and discarding the supernatant. The cell pellet was resuspended in 10 ml phosphate buffered saline and 1 ml of ^{111}In-oxine solution (20–40 MBq) was added drop by drop to the suspension of neutrophils. After incubation at room temperature for 15 minutes, 3 ml platelet poor plasma was added and the cell suspension was centrifuged at 250*g* for 10 minutes. The supernatant was discarded, the neutrophil cell pellet was resuspended to a total volume of 5 ml with equal parts of phosphate buffered saline and platelet poor plasma and the labelled cells were then ready for reinjection.

PATIENT IMAGING

All patients were injected at a fresh site with ^{111} labelled autologous neutrophils within two and half hours of the initial venesection. Prelimina studies had suggested that the optimal time f imaging was 24 hours after the injection of labell neutrophils and we used this imaging time in all o patients. Thus the earliest imaging time for a patient was 36 hours after the onset of chest pain a the latest was 57 hours. Each patient was imag while supine and planar images were acquired in t anterior, left anterior oblique, and left lateral po tion for 100 000 counts with a gamma cam (GE400 AT). In 24 patients single photon emissi computed tomography was performed with the sa gamma camera linked to a DEC PDP11/23+ co puter that used locally written software. T minutes before the single photon emiss computed tomography study, 40 MBq technetium-99 m (^{99m}Tc) human serum albur was administered to allow blood pool imaging sequence of 64 simultaneous images of ^{99m}Tc a ^{111}In was then acquired as the head of the gam camera rotated through 180°, starting in the ri anterior oblique position. The total imaging ti was 32 minutes. At the end of this per computerised reconstruction of the images performed.

IMAGE INTERPRETATION

An observer who was unaware of elec cardiographic findings or the maximum crea kinase rise graded planar and single photon emis computed tomography images as positive (in wl indium activity was clearly seen in the region of heart) or negative (where there was no detect

Table 1 *Patient details and results of imaging with indium-111 labelled autologous neutrophils*

	Positive image (n = 23)	*Negative image (n = 7)*	*Statistical significance*
Age (years)	62·0 (10·8)	59·8 (9·4)	NS (1)
Sex	(6F; 17M)	(2F; 5M)	NS (2)
Location of acute myocardial infarct	10 inf: 13 ant	3 inf: 4 ant	NS (2)
Interval from onset of chest pain to injection of ^{111}In neutrophils (h)	20·3 (6·4)	27·6 (5·8)	$p < 0·02$ (3)
Peak creatine kinase (u/l)	2023·5 (916·0)	1825 (1214·0)	NS (1)
Total leucocyte count (10^9/l)	12·9 (3·2)	12·5 (3·4)	NS (1)
Number of neutrophils injected ($\times 10^8$)	2·7 (0·9)	2·6 (0·7)	NS (1)
Activity of ^{111}In administered (MBq)	32·8 (8·4)	29·4 (8·2)	NS (1)
Drugs administered:			
Non-steroidal anti-inflammatory drugs	5	1	
Lignocaine	2	3	
Calcium antagonists	1	2	
Nitrates	7	1	

All values are shown as mean (SD).
(1) Unpaired *t* test.
(2) Exact probability test.
(3) Unpaired Wilcoxon rank sum test.

activity or where there was an area of activity on planar imaging that was inseparable from the liver, spleen, or ribs). Dual isotope single photon emission computed tomography images were considered to be positive when indium activity was seen in all three reconstruction views and corresponded with the ^{99m}Tc blood pool image.

STATISTICAL ANALYSIS

Data from the groups were compared by means of an unpaired *t* test, exact probability test, or unpaired Wilcoxon rank sum test as appropriate. Values of $p > 0{\cdot}05$ were regarded as not significant.

Results

In 23 of the 30 patients with acute myocardial infarction there was uptake of ^{111}In labelled neutrophils within the myocardium. Three patterns of cardiac uptake were seen[5]: focal myocardial uptake (12 patients), diffuse myocardial uptake (3 patients), and "doughnut pattern" (3 patients). Figure 1 shows an example of myocardial uptake. Planar images from all patients were graded as unequivocally positive or negative. In six patients in whom the planar images were considered to be negative, single photon emission computed tomography reconstruction showed localised uptake within the myocardium (fig 2). In addition, dual isotope single photon emission computed tomography reconstruction improved anatomical localisation of the infarct by confirming uptake corresponding to the cardiac blood pool (fig 3). There is a significant difference between the intervals from onset of chest pain to injection of labelled neutrophils in the groups with positive and negative images ($p < 0{\cdot}02$) (table 1). Furthermore, all patients reinjected within 18 hours had positive images whereas positive images were increasingly less common in those injected at progressively later intervals. Other features such as age, sex, peak creatine kinase, peripheral white blood cell count, dose of ^{111}In administered, and drug treatment did not influence the imaging results (table 1).

Discussion

This study confirms that ^{111}In labelled autologous neutrophils can be used to image the inflammatory response to acute myocardial infarction in man. The increased frequency of positive images in this study (77% compared with the 58% as previously described[8]) is in part related to earlier reinjection of ^{111}In labelled neutrophils after the onset of chest pain. This suggests that the stimulus for activation and migration of neutrophils to the area of myocardial damage is transient. This temporal relation may be of particular relevance because of the current interest in reducing the extent of myocardial damage after myocardial ischaemia or infarction by the use of intravenous thrombolytic treatment[10 11] or the potential for the administration of lipoxygenase or cycloxygenase inhibitors.[3]

Thrombolytic treatment can produce coronary reperfusion and hence it could improve myocardial salvage, but it may result in other events which in themselves are potentially harmful.[12] In particular, if the inflammatory response is exaggerated as a

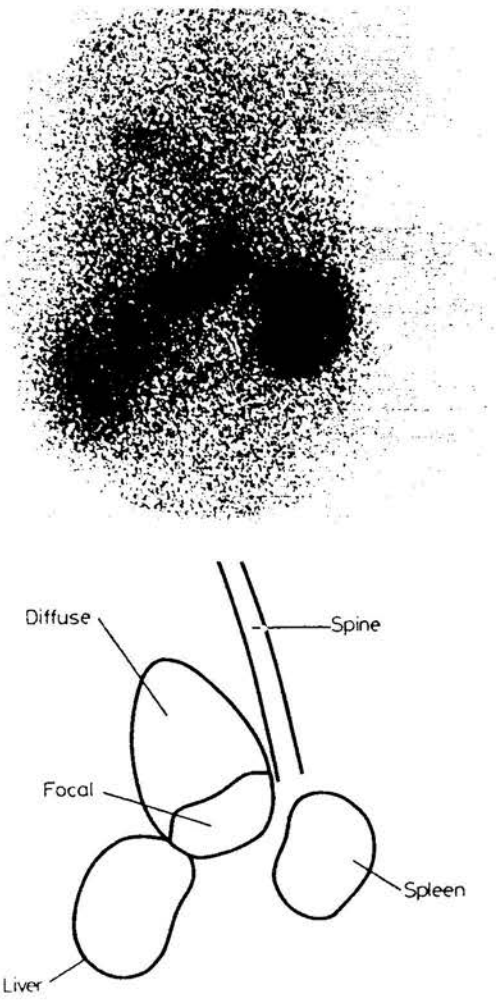

Fig 1 *Planar image in the left anterior oblique view showing normal uptake of ^{111}In labelled neutrophils in the liver and spleen and diffuse uptake in the region of the heart with an area of focal uptake in the inferior wall of the left ventricle. The line drawing shows the areas of uptake.*

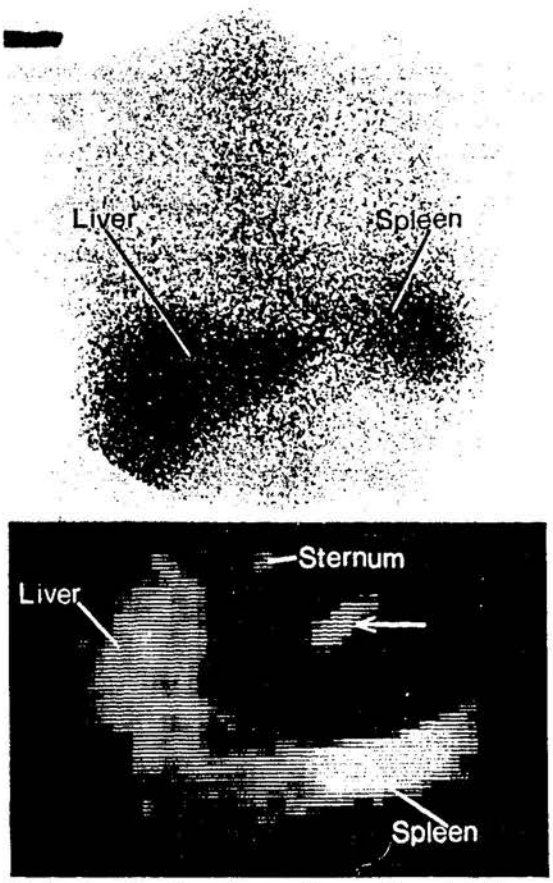

Fig 2 *Anterior planar image with normal uptake in liver and spleen and no definite myocardial uptake (top). Single photon emission computed tomographic image (bottom) in the transverse plane showing uptake within liver and spleen and an area of focal myocardial uptake (arrowed).*

result of neutrophils entering ischaemic tissue in greater numbers after reperfusion, the activated neutrophils could generate a number of cytotoxic products including oxygen derived free radicals and proteolytic enzymes, which can extend myocardial damage.[13] Both neutrophil depletion and the use of

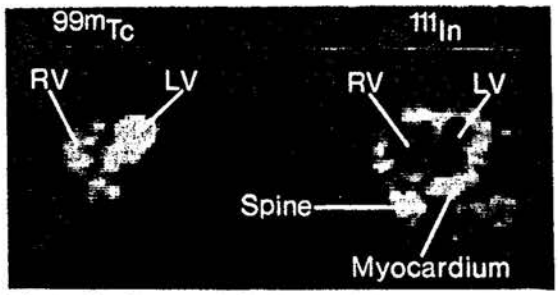

Fig 3 *Simultaneous single photon emission computed tomographic images in the transverse plane. The ^{99m}Tc image shows blood pool in the left and right ventricle. The corresponding ^{111}In image shows extensive uptake within the myocardium of both ventricles.*

non-steroidal anti-inflammatory drugs have been shown to reduce infarct size in experimental myocardial infarction,[3 4] and these findings may indicate further potential methods of improving myocardial salvage in the post-infarct period.

Single photon emission computed tomography increased the number of positive images by allowing spatial separation of positive myocardial uptake of indium from adjacent bone, liver, and spleen. It is not possible to ascertain from the planar images whether the ^{111}In activity detected in the region of the heart is due to blood pool activity. Dual isotope single photon emission computed tomography with ^{99m}Tc human serum albumin as a marker of blood pool, however, unequivocally demonstrated that the ^{111}In activity was localised in the myocardium. Single photon emission computed tomography, like pyrophosphate scans,[14] may also provide a method of quantifying neutrophil uptake within the myocardium.

Imaging with ^{111}In labelled autologous neutrophils in patients with acute myocardial infarction allows us to image the acute inflammatory response to myocardial damage, but should not be regarded as a technique for the diagnosis or localisation of acute myocardial infarction because other techniques are currently more successful. If inhibition of neutrophil migration limits the extent of myocardial

Table 2 *Imaging results*

		Positive images		
Time (h)	*Number of patients*	*Planar positive SPECT positive*	*Planar negative SPECT positive*	*Positive images %*
18	9	7	2	100
18–24	12	7	3	83
24–36	9	3	1	44

SPECT, single photon emission computed tomography.

infarction this method can be used to monitor the temporal relation and extent of neutrophil uptake in acute infarction.

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The use of ^{111}In-labelled autologous neutrophils in imaging myocardial infarction

A.J. MUIR, D. BELL, M. JACKSON, J.J. NICOLL and A.M. MILLAR

Department of Medicine and Medical Physics, Royal Infirmary, Edinburgh EH3 9YW, Scotland

Following myocardial infarction there is an acute inflammatory response, characterized by the migration of neutrophils into the area of infarcted muscle. Histological examination shows neutrophil infiltration into the infarcted area within 24 h and the response appears to be maximal at about 4 days [1]. Although neutrophil infiltration is part of the normal inflammatory response, in certain circumstances it might promote further autolytic changes. Neutrophils, when activated, can release oxygen-derived free radicals and proteolytic enzymes that may increase tissue injury, and the neutrophil has been implicated in a number of disease processes, including emphysema, the adult respiratory distress syndrome and even rheumatoid arthritis. A number of animal models of myocardial infarction have shown that infarct size can be limited by neutrophil inhibition [2, 3]. There is no evidence whether the neutrophil extends myocardial damage in man. Reperfusion increases neutrophil influx at the site of tissue injury with susbsequent activation which can disrupt the cell membranes leading to cell death. At the same time complement (C5a) is activated and this amplifies the neutrophil response which may cause local plugging of capillary tissue with neutrophils leading to further local tissue ischaemia and oedema. The introduction of thrombolytic therapy to reopen occluded coronary arteries has led to increased speculation about whether the advantages of revascularization might be partly offset by the disadvantages of revascularization injury.

To explore further the role of the neutrophils in the acute inflammatory response to myocardial infarction we have used indium-labelled autologous neutrophils. Although a number of methods existed to isolate pure neutrophils from blood, they were often time consuming and involved several steps. Using monopoly resolving medium we can isolate neutrophils from whole blood in a single step and, having proved the viability of this preparation, we can then examine its use in acute myocardial infarction [4].

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Methods

Cell separation and labelling procedures

Venous blood was collected aseptically into a syringe containing 300 units of preservative-free heparin. In duplicate, 25 ml of blood was layered over 12 ml of monopoly resolving medium (Flow laboratories) in a sterile tube and centrifuged at 400 *g* for 60 min. Differential migration during centrifugation results in two distinct cell bands and a red cell pellet. From the top plasma layer 8 ml was collected and centrifuged at 1000 *g* for 10 min to provide platelet-poor plasma (PPP). The remaining plasma and the upper cell band containing mononuclear cells were discarded. The neutrophils were recovered from the lower cell band and resuspended in 40 ml of phosphate-buffered saline (pH 7.4) and then centrifuged at 400 *g* for 10 min. The supernatant was discarded and the cell pellet was resuspended in 10 ml of the buffered saline. ^{111}In oxine solution (20–40 MBq) (Amersham International plc) was added dropwise to the suspension of neutrophils. After incubation at room temperature for 15 min, 3 ml of PPP was added and the cell suspension was centrifuged at 250 *g* for 10 min. The cell pellet was then resuspended to a volume of 5 ml with equal parts of phosphate-buffered saline and PPP.

Validation of labelling procedure

From 45 patients blood samples were taken in which separation was performed; neutrophil recovery was 47% and labelling efficiency was 72%. Red cell contamination was approximately 6% and lymphocyte contamination was less than 0.5%. In eight patients without heart or lung disease, kinetic data showed that lung count rates fell to 19% of the maximum whole field count rate within the first 90 s and thereafter fell at a lower rate to 9% by 20 min, indicating only a small degree of lung retention. Heart count rates fell to 3% of the maximum whole field count rate within 90 s and remained constant at 2% for the remainder of the study. Liver count rates rose to 16% by 15 min and remained constant for the rest of the study period whilst splenic counts continued to rise slowly throughout the study. Of 45 patients studied with suspected occult infection, 18 showed a positive uptake. No patient with a negative scan was subsequently demonstrated to have a major site of infection.

Imaging and myocaridal infarction

We examined 30 patients who had sustained a recent acute myocardial infarction [5]. The location of the acute myocardial infarction was inferior in 13 and anterior in 17. All patients were reinjected at a fresh site with ^{111}In-labelled autologous neutrophils within 2.5 h of the initial venesection. The time from onset of symptoms to reinjection was noted. Imaging took place 6 and 24 h after reinjection of the labelled neutrophils. The 24 h images proved superior to those taken at 6 h and we have therefore used the 24 h images for data analysis. Thus the earliest 'imaging time' for any patient was 36 h after the onset of chest pain and the latest 57 h. Each patient was imaged while supine, and planar images were acquired in the anterior, left anterior oblique and left lateral position from 100 000 counts with a gamma camera (GE 400 AT). In 24 patients single photon emission computed tomography was performed with the same camera linked to a DEC PDP 11/23 plus computer using locally written software. Ten minutes before the single photon emission computed tomography study, 40 MBq of ^{99}Tc^{m}-labelled human serum albumin was administered to allow blood pool imaging for accurate anatomical location. A sequence of 64 simultaneous images of ^{99}Tc^{m} and ^{111}In was then acquired as the head of the gamma camera rotated through 180°, starting in the right anterior oblique position. The total imaging time was 32 min.

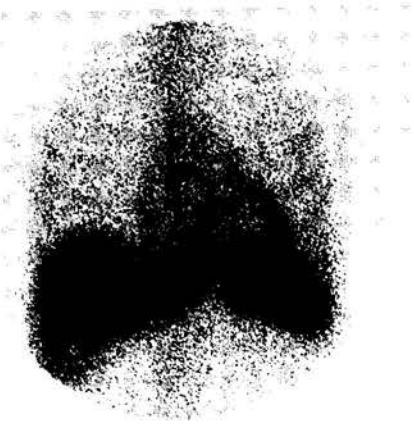

Fig. 1. Anterior planar image illustrating uptake of indium-labelled neutrophils in liver, spleen and myocardium. This patient had sustained a recent anterior myocardial infarction.

Results

In 23 of the 30 patients with acute myocardial infarction there was uptake of ^{111}In-labelled neutrophils within the myocardium (Fig. 1). In six patients in whom the planar images were considered to be negative, single photon emission computed tomography reconstruction showed localized uptake within the myocardium. The peak creatine kinase activity, the total leucocyte count, the number of neutrophils reinjected and the activity of ^{111}In administered all had no influence on whether there was a positive or negative image. In the same way there seemed to be no relation between the drugs the patient was receiving and the uptake or lack of uptake of neutrophils in the infarcted myocardium. The only factor we could determine that influenced the uptake of neutrophils was the time from the onset of chest pain (Fig. 2). All patients reinjected within 18 h had positive images, whereas positive images were increasingly less common in those injected at progressively later intervals.

Discussion

These studies confirm that a pure population of neutrophils can be isolated from whole blood without the need to perform an initial sedimentation step using dextran, hydroxyethylstarch or methylcellulose as is often used. Subsequent labelling of the separated pure neutrophils is achieved with ^{111}In oxinate. Our method does not require the

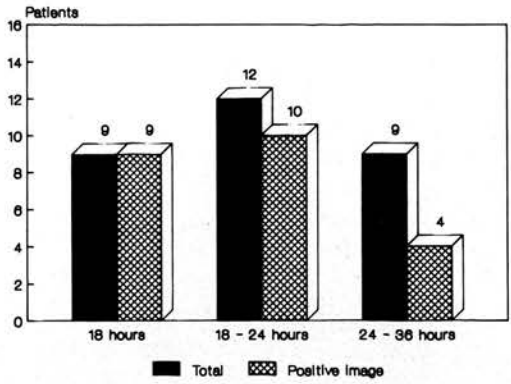

Fig. 2. The incidence of positive imaging with indium-labelled neutrophils after acute myocardial infarction. Where labelling and reinjection took place within 18 h of the onset of symptoms, all patients had a positive image, 10 of the 12 had positive images if reinjection took place within 24 h, but only four of nine had positive images where reinjection took place more than 24 h after the onset of symptoms.

preparation of a discontinuous density gradient but uses the commercially available monopoly resolving medium. Good neutrophil recovery is achieved with little red cell and virtually no lymphocyte contamination. There is no evidence of lung retention or liver sequestration of these labelled cells, and, as they identify sites of infection, they appear to be functionally normal.

Using these autologous labelled neutrophils we have demonstrated the inflammatory response to acute myocardial infarction in man. The increased frequency of positive images in our study (77% compared with the 58% previously described by Davies and colleagues [6]) seem to relate to the early reinjection of the labelled neutrophils after the onset of chest pain. We would suggest that the stimulus for activation and migration of neutrophils to the area of myocardial damage is early. This must be contrasted with the evidence from pathological studies where neutrophil infiltration appears to be maximum 2–4 days after the onset of symptoms.

Neutrophil depletion and the inhibition of the inflammatory response have been shown to reduce infarct size in experimental myocardial infarction, and our results suggest that, if modulation of the inflammatory response is to be undertaken in man, this technique is capable of identifying alterations in neutrophil uptake. Imaging with ^{111}In-labelled autologous neutrophils in patients with acute myocardial infarction allows a study of the acute inflammatory response to myocardial damage, but should not be regarded as a technique for the diagnosis or localization of acute myocardial infarction because other techniques are currently more successful.

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